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<b>(54) Title:</b> NON-STOCHASTIC GENERATION OF GENETIC VACCINES AND ENZYMES  <b>(57) Abstract</b>  This invention provides methods of obtaining novel polynucleotides and encoded polypeptides by use of non-stochastic methods of directed evolution (DirectEvolution™). These methods include non-stochastic polynucleotide site-saturation mutagenesis (Gene Site Saturation Mutagenesis™) and non-stochastic polynucleotide reassembly (GeneReassembly™). Through use of the claimed methods, genetic vaccines, enzymes, and other desirable molecules can be evolved towards desirable properties. For example, vaccine vectors can be obtained that exhibit increased efficacy for use as genetic vaccines. Vectors obtained by using the methods can have, for example, enhanced antigen expression, increased uptake into a cell, increased stability in a cell, ability to tailor an immune response, and the like. This invention provides methods of obtaining novel enzymes that have optimized physical and/or biological properties. Furthermore, this invention provides methods of obtaining a variety of novel biologically active molecules, in the fields of antibiotics, pharmacotherapeutics, and transgenic traits.		

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# NON-STOCHASTIC GENERATION OF GENETIC VACCINES AND ENZYMES

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## 1. GENERAL

### 1.1. FIELD OF THE INVENTION

5           This invention pertains to the field of genetic vaccines. Specifically, the invention provides multi-component genetic vaccines that contain components that are optimized for a particular vaccination goal. In a particular aspect this invention provides methods for improving the efficacy of genetic vaccines by providing materials that facilitate targeting of a genetic vaccine to a particular  
10   tissue or cell type of interest.

          This invention also pertains to the field of modulation of immune responses such as those induced by genetic vaccines and also pertains to the field of methods for developing immunogens that can induce efficient immune  
15   responses against a broad range of antigens.

          Thus, the present invention also relates generally to novel proteins, and fragments thereof, as well as nucleic acids which encode these proteins, and methods of making and using these proteins in diagnostic, prophylactic and  
20   therapeutic applications. In a particular exemplification, the present invention relates to proteins from the Plasmodium falciparum erythrocyte membrane protein 1 ("PfEMP1") gene family and fragments thereof which are derived from malaria parasitized erythrocytes. In particular, these proteins are derived from the erythrocyte membrane protein of Plasmodium falciparum parasitized  
25   erythrocytes, also termed "PfEMP1". The present invention also provides nucleic acids encoding these proteins, which proteins and nucleic acids are associated with the pathology of malaria infections, and which may be used as vaccines or other prophylactic treatments for the prevention of malaria infections, and/or in

diagnosing and treating the symptoms of patients who suffer from malaria and associated diseases.

This invention also relates to the field of protein engineering. Specifically,  
5 this invention relates to a directed evolution method for preparing a  
polynucleotide encoding a polypeptide. More specifically, this invention relates  
to a method of using mutagenesis to generate a novel polynucleotide encoding a  
novel polypeptide, which novel polypeptide is itself an improved biological  
molecule &/or contributes to the generation of another improved biological  
10 molecule. More specifically still, this invention relates to a method of performing  
both non-stochastic polynucleotide chimerization and non-stochastic site-directed  
point mutagenesis.

Thus, in one aspect, this invention relates to a method of generating a  
15 progeny set of chimeric polynucleotide(s) by means that are synthetic and non-  
stochastic, and where the design of the progeny polynucleotide(s) is derived by  
analysis of a parental set of polynucleotides &/or of the polypeptides  
correspondingly encoded by the parental polynucleotides. In another aspect this  
invention relates to a method of performing site-directed mutagenesis using  
20 means that are exhaustive, systematic, and non-stochastic.

Furthermore this invention relates to a step of selecting from among a  
generated set of progeny molecules a subset comprised of particularly desirable  
species, including by a process termed end-selection, which subset may then be  
25 screened further. This invention also relates to the step of screening a set of  
polynucleotides for the production of a polypeptide &/or of another expressed  
biological molecule having a useful property.

Novel biological molecules whose manufacture is taught by this invention include genes, gene pathways, and any molecules whose expression is affected thereby, including directly encoded polypeptides &/or any molecules affected by such polypeptides. Said novel biological molecules include those that contain a  
5 carbohydrate, a lipid, a nucleic acid, &/or a protein component, and specific but non-limiting examples of these include antibiotics, antibodies, enzymes, and steroidal and non-steroidal hormones.

In a particular non-limiting aspect, the present invention relates to  
10 enzymes, particularly to thermostable enzymes, and to their generation by directed evolution. More particularly, the present invention relates to thermostable enzymes which are stable at high temperatures and which have improved activity at lower temperatures.

15

## 1.2. BACKGROUND

Providing protective immunity even in situations when the pathogens are  
20 poorly characterized or cannot be isolated or cultured in laboratory  
environment.

Genetic immunization represents a novel mechanism of inducing protective humoral and cellular immunity. Vectors for genetic vaccinations  
25 generally consist of DNA that includes a promoter/enhancer sequence, the gene of interest and a polyadenylation/ transcriptional terminator sequence. After intramuscular or intradermal injection, the gene of interest is expressed, followed by recognition of the resulting protein by the cells of the immune system. Genetic immunizations provide means to induce protective immunity even in situations

when the pathogens are poorly characterized or cannot be isolated or cultured in laboratory environment.

5    **Small improvement in the efficiency of genetic vaccine vectors can result in dramatic increase if the level of immune response**

10    The efficacy of genetic vaccination is often limited by inefficient uptake of genetic vaccine vectors into cells. Generally, less than 1% of the muscle or skin cells at the sites of injections express the gene of interest. Even a small improvement in the efficiency of genetic vaccine vectors to enter the cells can result in a dramatic increase in the level of immune response induced by genetic vaccination. A vector typically has to cross many barriers which can result in only a very minor fraction of the DNA ever being expressed.

15

**Various limitations to immunogenicity**

20    Limitations to immunogenicity include: loss of vector due to nucleases present in blood and tissues; inefficient entry of DNA into a cell; inefficient entry of DNA into the nucleus of the cell and preference of DNA for other compartments; lack of DNA stability in the nucleus (factor limiting nuclear stability may differ from those affecting other cellular and extracellular compartments), and, for vectors that integrate into the chromosome, the efficiency of integration and the site of integration. Moreover, for many applications of genetic vaccines, it is preferable for the genetic vaccine to enter a particular target tissue or cell.

25

Thus, a need exists for genetic vaccines that can be targeted to specific cell and tissue types of interest, and which exhibit an increased ability to enter the target cells. The present invention fulfills these and other needs.

5

**Pathways for immune responses induced by genetic vaccines**

Elicitation of a desired *in vivo* response by a genetic vaccine generally requires multiple cellular processes in a complex sequence. Several potential pathways exist along which a genetic vaccine can exert its effect on the mammalian immune system. In one pathway, the genetic vaccine vector enters cells that are the predominant cell type in the tissue that receives vaccine (e.g., muscle or epithelial cells). These cells express and release the antigen encoded by the vector. The vaccine vector can be engineered to have the antigen released as an intact protein from living transfected cells (i.e., via a secretion process) or directed to a membrane-bound form on the surface of these cells. Antigen can also be released from an intracellular compartment of such cells if those cells die.

**The antigen derived from vaccine vector internalization and antigen expression within the predominant cell type in the tissue ends up within APC, which then process the antigen internally to prime MHC Class I and or Class II, essential steps in activation of CD4<sup>+</sup> T-helper cells and development of potent specific immune responses.**

25

Extracellular antigen derived from any of these situations interacts with antigen presenting cells (APC) either by binding to the cell surface (specifically via IgM or via other non-immunoglobulin receptors) and subsequent endocytosis of outer membrane, or by fluid phase micropinocytosis wherein the APC

internalizes extracellular fluid and its contents into an endocytic compartment. Interaction with APC may occur before or after partial proteolytic cleavage in the extracellular environment. In any case, the antigen derived from vaccine vector internalization and antigen expression within the predominant cell type in the tissue ends up within APC. The APC then process the antigen internally to prime MHC Class I and or Class II, essential steps in activation of CD4<sup>+</sup> T-helper cells (T<sub>H</sub>1 and/or T<sub>H</sub>2) and development of potent specific immune responses.

10 **The genetic vaccine plasmid enters APC and antigen is proteolytically cleaved in the cell cytoplasm.**

In a parallel pathway, the genetic vaccine plasmid enters APC (or the predominant cell type in the tissue) and, instead of antigen derived from plasmid expression being directed to extracellular export, antigen is proteolytically cleaved in the cell cytoplasm (in a proteasome dependent or independent process). Often, intracellular processing in such cells occurs via proteasomal degradation into peptides that are recognized by the TAP-1 and TAP-2 proteins and transported into the lumen of the rough endoplasmic reticulum (RER).

20

**The peptide fragments are transported into the RER complex, expressed on the cell surface; in the presence of appropriate additional signals, can differentiate into functional CTLs.**

25

The peptide fragments transported into the RER complex with MHC Class I. Such antigen fragments are then expressed on the cell surface in association with Class I. CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) bearing specific T cell

receptor then recognize the complex and can, in the presence of appropriate additional signals, differentiate into functional CTLs.

5            **By virtue of poorly characterized pathways for trafficking of cytoplasmically generated peptides into endosomal compartments, a genetic vaccine vector can lead to CD4<sup>+</sup> T cell stimulation.**

10            In addition, poorly characterized pathways, which are generally not dominant, exist in APC for trafficking of cytoplasmically generated peptides into endosomal compartments where they can end up complexed with MHC Class II, and thereby act to present antigen peptides to CD4<sup>+</sup> T<sub>H</sub>1 and T<sub>H</sub>2 cells. Because activation, proliferation, differentiation and immunoglobulin isotype switching by B lymphocytes requires help of CD4<sup>+</sup> T cells, antigen presentation in the context  
15            of MHC Class II molecules is crucial for induction of antigen-specific antibodies. By virtue of this pathway, a genetic vaccine vector can lead to CD4<sup>+</sup> T cell stimulation in addition to the dominant CD8<sup>+</sup> CTL activation process described above. This alternative pathway is, however, of little consequence in muscle cells where levels of MHC Class II expression are very low or zero.

20

**In this case cytokines are derived not only from processes intrinsic to the interaction of DNA with cells, or specific cell responses to the antigen, but via synthesis directed by the vaccine plasmid.**

25

Genetic vaccination can also elicit cytokine release from cells that bind to or take up DNA. So-called immunostimulatory or adjuvant properties of DNA are derived from its interaction with cells that internalize DNA. Cytokines can be released from cells that bind and/or internalize DNA in the absence of gene



transcription. Separately, interaction of antigen with APC followed by presentation and specific recognition also stimulates release of cytokines that have positive feedback effects on these cells and other immune cells. Chief among these effects are the direction of  $CD4^+$   $T_H$  cells to differentiate/ proliferate preferentially to  $T_H1$  or  $T_H2$  phenotypes. Furthermore, cytokines released at the site of DNA vaccination, regardless of the mechanism of their release, contribute to recruitment of other immune cells from the immediate local area and more distant sites such as draining lymph nodes. In recognition of the importance of cytokines in elicitation of a potent immune response, some investigators have included the genes for one or more cytokines in the DNA vaccine plasmid along with the target antigen for immunization. In this case cytokines are derived not only from processes intrinsic to the interaction of DNA with cells, or specific cell responses to the antigen, but via synthesis directed by the vaccine plasmid.

15

**Movement of immune cells from the blood stream and different sites to the site of immunization and also from the site of immunization to other sites**

Immune cells are recruited to the site of immunization from distant sites or the bloodstream. Specific and non-specific immune responses are then greatly amplified. Immune cells, including APC, bearing antigen fragments complexed to MHC molecules or even expressing antigen from uptake of plasmid, also move from the immunization site to other sites (blood, hence to all tissues; lymph nodes; spleen) where additional immune recruitment and qualitative and quantitative development of the immune response ensue.

Current genetic vaccine vectors employ simple methods for expression of the desired antigen with few if any design elements that control the precise intracellular fate of the antigen or the immunological consequences of antigen expression

5

While these pathways often compete, previously available genetic vaccines have incorporated all components for influencing each of the pathways into a single polynucleotide molecule. Because separate cell types are involved in the complex interactions required for a potent immune response to a genetic vaccine vector, mutually incompatible consequences can arise from administration of a genetic vaccine that is incorporated in a single vector molecule. Current genetic vaccine vectors employ simple methods for expression of the desired antigen with few if any design elements that control the precise intracellular fate of the antigen or the immunological consequences of antigen expression. Thus, although genetic vaccines show great promise for vaccine research and development, the need for major improvements and several severe limitations of these technologies are apparent.

20 Existing genetic vaccine vectors have not been optimized for human tissue, providing low and short-lasting expression of the antigen of interest, with insufficient stability, inducibility, or levels of expression *in vivo*, among other things

25 Largely due to the lack of suitable laboratory models, none of the existing genetic vaccine vectors have been optimized for human tissues. The existing genetic vaccine vectors typically provide low and short-lasting expression of the antigen of interest, and even large quantities of DNA do not always result in sufficiently high expression levels to induce protective immune responses.

Because the mechanisms of the vector entry into the cells and transfer into the nucleus are poorly understood, virtually no attempts have been made to improve these key properties. Similarly, little is known about the mechanisms that regulate the maintenance of vector functions, including gene expression. Furthermore,  
5 although there is increasing amount of data indicating that specific sequences alter the immunostimulatory properties of the DNA, rational engineering is a very laborious and time- consuming approach when using this information to generate vector backbones with improved immunomodulatory properties.

10 Moreover, presently available genetic vaccine vectors do not provide sufficient stability, inducibility or levels of expression *in vivo* to satisfy the desire for vaccines which can deliver booster immunization without additional vaccine administration. Booster immunizations are typically required 3-4 weeks after the primary injection with existing genetic vaccines.

15 Therefore a need exists for improved genetic vaccine vectors and formulations, and methods for development of such vectors. The present invention fulfills these and other needs.

20 The interactions between pathogens and hosts are results of millions of years of evolution, during which the mammalian immune system has evolved sophisticated means to counterattack pathogen invasions. However, bacterial and viral pathogens have simultaneously gained a number of mechanisms to improve their virulence and survival in hosts, providing a major challenge for vaccine  
25 research and development despite the powers of modern techniques of molecular and cellular biology. Similar to the evolution of pathogen antigens, several cancer antigens are likely to have gained means to downregulate their immunogenicity as a mechanism to escape the host immune system.

Efficient vaccine development is also hampered by the antigenic heterogeneity of different strains of pathogens, driven in part by evolutionary forces as means for the pathogens to escape immune defenses. Pathogens also reduce their immunogenicity by selecting antigens that are difficult to express, process and/or transport in host cells, thereby reducing the availability of immunogenic peptides to the molecules initiating and modulating immune responses. The mechanisms associated with these challenges are complex, multivariate and rather poorly characterized. Accordingly, a need exists for vaccines that can induce a protective immune response against bacterial and viral pathogens. The present invention fulfills this and other needs.

Antigen processing and presentation is only one factor which determines the effectiveness of vaccination, whether performed with genetic vaccines or more classical methods. Other molecules involved in determining vaccine effectiveness include cytokines (interleukins, interferons, chemokines, hematopoietic growth factors, tumor necrosis factors and transforming growth factors), which are small molecular weight proteins that regulate maturation, activation, proliferation and differentiation of the cells of the immune system.

Characteristic features of cytokines are pleiotropy and redundancy; that is, one cytokine often has several functions and a given function is often mediated by more than one cytokine. In addition, several cytokines have additive or synergistic effects with other cytokines, and a number of cytokines also share receptor components.

Due to the complexity of the cytokine networks, studies on the physiological significance of a given cytokine have been difficult, although recent studies using cytokine gene-deficient mice have significantly improved our understanding on the functions of cytokines *in vivo*. In addition to soluble

proteins, several membrane- bound costimulatory molecules play a fundamental role in the regulation of immune responses. These molecules include CD40, CD40 ligand, CD27, CD80, CD86 and CD150 (SLAM), and they are typically expressed on lymphoid cells after activation via antigen recognition or through cell-cell interactions.

T helper (T<sub>H</sub>) cells, key regulators of the immune system, are capable of producing a large number of different cytokines, and based on their cytokine synthesis pattern T<sub>H</sub> cells are divided into two subsets (Paul and Seder (1994) *Cell* 76: 241-251). T<sub>H</sub>1 cells produce high levels of IL-2 and IFN- $\gamma$  and no or minimal levels of IL-4, IL-5 and IL-13. In contrast, T<sub>H</sub>2 cells produce high levels of IL-4, IL-5 and IL-13, and IL-2 and IFN- $\gamma$  production is minimal or absent. T<sub>H</sub>1 cells activate macrophages, dendritic cells and augment the cytolytic activity of CD8<sup>+</sup> cytotoxic T lymphocytes and NK cells (*Id.*), whereas T<sub>H</sub>2 cells provide efficient help for B cells and they also mediate allergic responses due to the capacity of T<sub>H</sub>2 cells to induce IgE isotype switching and differentiation of B cells into IgE secreting cell (De Vries and Punnonen (1996) In *Cytokine regulation of humoral immunity: basic and clinical aspects*. Eds. Snapper, C.M., John Wiley & Sons, Ltd., West Sussex, UK, p. 195- 215). The exact mechanisms that regulate the differentiation of T helper cells are not fully understood, but cytokines are believed to play a major role. IL-4 has been shown to direct T<sub>H</sub>2 differentiation, whereas IL-12 induces development of T<sub>H</sub>1 cells (Paul and Seder, *supra.*). In addition, it has been suggested that membrane bound costimulatory molecules, such as CD80, CD86 and CD150, can direct T<sub>H</sub>1 and/or T<sub>H</sub>2 development, and the same molecules that regulate T<sub>H</sub> cell differentiation also affect activation, proliferation and differentiation of B cells into Ig-secreting plasma cells (Cocks *et al.* (1995) *Nature* 376: 260-263; Lenschow *et al.* (1996) *Immunity* 5: 285-293; Punnonen *et al.* (1993) *Proc. Nat'l. Acad. Sci. USA* 90: 3730-3734; Punnonen *et al.* (1997) *J Exp. Med.* 185: 993-1004).

Studies in both man and mice have demonstrated that the cytokine synthesis profile of T helper (T<sub>H</sub>) cells plays a crucial role in determining the outcome of several viral, bacterial and parasitic infections. High frequency of T<sub>H</sub>1 cells generally protects from lethal infections, whereas dominant T<sub>H</sub>2 phenotype often results in disseminated, chronic infections. For example, T<sub>H</sub>1 phenotype is observed in tuberculoid (resistant) form of leprosy and T<sub>H</sub>2 phenotype in lepromatous, multibacillary (susceptible) lesions (Yamamura *et al.* (1991) *Science* 254: 277-279). Similarly, late-stage HIV patients have T<sub>H</sub>2-like cytokine synthesis profiles, and T<sub>H</sub>1 phenotype has been proposed to protect from AIDS (Maggi *et al.* (1994) *J Exp. Med.* 180: 489-495). Furthermore, the survival from meningococcal septicemia is genetically determined based on the capacity of peripheral blood leukocytes to produce TNF- $\alpha$  and IL-10. Individuals from families with high production of IL-10 have increased risk of fatal meningococcal disease, whereas members of families with high TNF- $\alpha$  production were more likely to survive the infection (Westendorp *et al.* (1997) *Lancet* 349: 170-173).

Cytokine treatments can dramatically influence T<sub>H</sub>1/T<sub>H</sub>2 cell differentiation and macrophage activation, and thereby the outcome of infectious diseases. For example, BALB/c mice infected with *Leishmania major* generally develop a disseminated fatal disease with a T<sub>H</sub>2 phenotype, but when treated with anti-IL-4 mAbs or IL-12, the frequency of T<sub>H</sub>1 cells in the mice increases and they are able to counteract the pathogen invasion (Chatelain *et al.* (1992) *J Immunol.* 148: 1182-1187). Similarly, IFN- $\gamma$  protects mice from lethal Herpes Simplex Virus (HSV) infection, and MCP-1 prevents lethal infections by *Pseudomonas aeruginosa* or *Salmonella typhimurium*. In addition, cytokine treatments, such as recombinant IL-2, have shown beneficial effects in human

common variable immunodeficiency (Cunningham-Rundles *et al.* (1994) *N. Engl. J Med.* 331: 918-921).

5       The administration of cytokines and other molecules to modulate immune  
responses in a manner most appropriate for treating a particular disease can  
provide a significant tool for the treatment of disease. However, presently  
available immunomodulator treatments can have several disadvantages, such as  
insufficient specific activity, induction of immune responses against, the  
immunomodulator that is administered, and other potential problems. Thus, a  
10   need exists for immunomodulators that exhibit improved properties relative to  
those currently available. The present invention fulfills this and other needs.

Erythrocytes infected with the malaria parasite *P. falciparum* disappear  
from the peripheral circulation as they mature from the ring stage to trophozoites  
15   (Bignami and Bastianeli, *Reforma Medica* (1889) 6:1334-1335). This  
phenomenon, known as sequestration, results from parasitized erythrocyte ("PE")  
adherence to microvascular endothelial cells in diverse organs (Miller, *Am. J.*  
*Trop. Med. Hyg.* (1969) 18:860-865). Sequestration is associated temporally with  
expression of knob protrusions (Leech *et al.*, *J. Cell. Biol.* (1984) 98:1256-1264),  
20   expression of a very large antigenically variant surface protein, called PfEMP1  
(Aley *et al.*, *J. Exp. Med.* (1984) 160:1585-1590; Leech *et al.*, *J. Exp. Med.* (1984)  
159:1567-1575; Howard *et al.*, *Molec. Biochem. Parasitol.* (1988) 27:207-223),  
and expression of new receptor properties which mediate adherence to endothelial  
cells (Miller, *supra*; Udeinya *et al.*, *Science* (1981) 213:555-557. Endothelial cell  
25   surface proteins such as CD36, thrombospondin (TSP) and ICAM-1 have been  
identified as major host receptors for mature PE. *See, e.g.*, Barnwell *et al.*, *J.*  
*Immunol.* (1985) 135:3494-3497; Roberts *et al.*, *Nature* (1985) 318:64-66; and  
Berendt *et al.*, *Nature* (1989) 341:57-59.

PE sequestration confers unique advantages for *P. falciparum* parasites (Howard and Gilladoga, *Blood* (1989) 74:2603-2618), but also contributes directly to the acute pathology of *P. falciparum* (Miller *et al.*, *Science* (1994) 264:1878-1883). Of the four human malaras, only *P. falciparum* infection is  
5 associated with neurological impairment and cerebral pathology seen increasingly in severe drug-resistant malaria (Howard and Gilladoga, *supra*).

Although the genesis of human cerebral malaria is likely due to a combination of factors including particular parasite phenotypes (Berendt *et al.*,  
10 *Parasitol. Today* (1994) 10:412- 414), inappropriate immune responses and the phenotype of endothelial cell surface molecules in the cerebral microvasculature (Pasloske and Howard, *Ann. Rev. Med.* (1994) :283-295), adherence of PE to cerebral blood vessels and consequent local microvascular occlusion is a major contributing factor. See, e.g., Berendt *et al.*, *supra*; Patnaik *et al.*, *Am. J. Trop.*  
15 *Med. Hyg.* (1994) 51:642-647.

The capacity of *P. falciparum* PE to express variant forms of PfEMP1 contributes to the special virulence of this parasite. Variant parasites can evade variant-specific antibodies elicited by earlier infections. The *P. falciparum* variant  
20 antigens have been defined *in vitro* using antiserum prepared in Aotus monkeys infected with individual parasite strains (Howard *et al.*, *Molec. Biochem. Parasitol.* (1988) 27:207-223). Antibodies raised against a particular parasite will only react by PE agglutination, indirect immuno- fluorescence or immunoelectronmicroscopy with PE from the same strain (van Schravendijk *et*  
25 *al.*, *Blood* (1991) 78:226-236).

Such studies with PE from malaria patients in diverse geographic locations and sera from the same or different patients confirm that PE in natural isolates express variant surface antigens and that individual patients respond to infection



- by production of isolate-specific antibodies (Marsh and Howard, *Science* (1986) 231:150-153; Aguiar et al., *Am. J. Trop. Med. Hyg.* (1992) 47:621-632; Iqbal et al., *Trans. R. Soc. Trop. Med. Hyg.* (1993) 87:583-588. Expression of a variant antigen on PE has also been demonstrated in several simian, murine and human
- 5 malaria species, including *P. knowlesi* (Brown and Brown, *Nature* (1965) 208:1286-1288; Barnwell et al., *Infect. Immun.* (1983) 40:985-994), *P. chabaudi* (Gilks et al., *Parasite Immunol.* (1990) 12:45-64; Brannan et al., *Proc. R. Soc. Lond. Biol. Sci.* (1994) 256:71-75), *P. fragile* (Handunnetti et al., *J. Exp. Med.* (1987) 165:1269-1283) and *P. vivax* (Mendis et al., *Am. J. Trop. Med. Hyg.* (1988) 38:42-46). Laboratory studies with *P. knowlesi* (Brown and Brown, *supra*;
- 10 Barnwell et al., *supra*) or *P. falciparum* (Hommel et al., *J. Exp. Med.* (1983) 157:1137-1148) in monkeys and *P. chabaudi* in mice (Gilks et al., *supra*) confirmed that antigenic variation at the PE surface is associated with prolonged or chronic infection and the capacity to repeatedly re-establish blood infection in
- 15 previously infected animals. Studies with cloned parasites demonstrated that antigenic variants can arise with extraordinary frequency, e.g., 2% per generation with *P. falciparum* (Roberts et al., *Nature* (1992) 357:689-692) and 1.6 % per generation with *P. chabaudi* (Brannan et al., *supra*).
- 20 PfEMP1 was identified as a <sup>125</sup>I-labeled, size diverse protein (200-350 kD) on PE that is lacking from uninfected erythrocytes, and that is also labeled by biosynthetic incorporation of radiolabeled amino acids (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575; Howard et al., *Molec. Biochem. Parasitol.* (1988) 27:207-223). PfEMP1 is not extracted from PE by neutral detergents such as Triton X-
- 25 100 but is extracted by SDS, suggesting that it is linked to the erythrocyte cytoskeleton (Aley et al., *J. Med. Exp.* (1984) 160:1585-1590). After addition of excess Triton X-100, PfEMP1 is immunoreactive with appropriate serum antibodies (Howard et al., (1988), *supra*). Mild trypsinization of intact PE rapidly cleaves PfEMP1 from the cell surface (Leech et al., *J. Exp. Med.* (1984)

- 159:1567-1575). PfEMP1 bears antigenically diverse epitopes since it is immunoprecipitated from particular strains of *P. falciparum* by antibodies from sera of Aotus monkeys infected with the same strain, but not by antibodies from animals infected with heterologous strains (Howard et al. (1988), supra).
- 5 Knobless PE derived from parasite passage in splenectomized Aotus monkeys (Aley et al., supra) do not express surface PfEMP1 and are not agglutinated with sera from immune individuals or infected monkeys (Howard et al. (1988), supra; Howard and Gilladoga, Blood (1989) 74:2603-2618). In general, sera that react with the PE surface by indirect immunofluorescence and antibody-mediated PE
- 10 agglutination are the only sera to immunoprecipitate <sup>125</sup>I-labeled PfEMP1 from any particular strain (Howard et al., (1988), supra; van Schravendijk et al., Blood (1991) 78:226- 236; Biggs et al., J. Immunol. (1992) 149:2047-2054).

- The adherence of parasitized erythrocytes to endothelial cells is mediated
- 15 by multiple receptor/counter- receptor interactions, including CD36, thrombospondin and intracellular adhesion molecule-1 (ICAM\_1) as the major host cell receptors (Howard and Gilladoga, Blood (1989) 74:2603- 2618, Pasloske and Howard, Ann. Rev. Med. (1994) 45:283-295).

- 20 Vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) have also been implicated as additional endothelial cell receptors that can mediate adherence of a minority of *P. falciparum* PE (Ockenhouse, et al., J. Exp. Med. (1992) 176:1183-1189, and Howard and Paslaske, supra). The adherence receptors on the surface of PE has
- 25 not yet been conclusively identified, and several molecules, including AG 332 (Udomsangpetch, et al., Nature (1989) 338:763-765), modified band 3 (Crandall, et al., Proc. Nat'l Acad. Sci. USA (1993) 90:4703-4707), Sequesterin (Ockenhouse, Proc. Nat'l Acad. Sci. USA (1991) 88:3175-3179), and PfEMP1 (Howard and Gilladoga, supra, and Pasloske and Howard, supra), have been proposed as

candidates. Several pieces of indirect evidence have linked expression of PfEMP1 with the acquisition of new host protein receptor properties on the surface of PE (Howard and Gilladoga, *supra*; Pasloske and Howard, *Ann. Rev. Med.* (1994) 45:283-295). PE adherence is correlated with the expression of PfEMP1 on the surface of mature stage PE (Leech, et al., *J. Exp. Med.* (1984) 159:1567- 1575). Alterations in the adherence phenotype of the PE selected for *in vitro* are usually associated with the emergence of new forms of PfEMP1 (Biggs, et al., *J. Immunol.* (1992) 149:2047-2054; Roberts, et al., *Nature* (1992) 357:689- 692). Mild trypsinization of intact mature PE cleaves the extracellular portion of PfEMP1 and at the same time, reduces or eliminates PE cytoadherence (Leech, et al., *supra*) Previously described antibody mediated blockade or reversal of cytoadherence is strain specific and is correlated with the ability of the reacting sera to agglutinate the corresponding PE and to immunoprecipitate the surface labeled <sup>125</sup>I-PfEMP1 (Howard, et al., *Molec. Biochem. Parasitol.* (1988) 27:207-224). Pfalhesin (modified band 3) have been shown to bind CD36 under non-physiological conditions (Crandall, et al., *Exp. Parasitol.* (1994) 78:203-209). Sequestrin, which appears to be homologous to PfEMP1, extracted with TX100 from knobless PE, was shown to bind to immobilized CD36 (Ockenhouse, *Proc. Nat'l Acad. Sci. USA* (1991) 88:3175-3179).

20

The complex nature and/or mechanism of malarial antigenic variation, and its particular virulence has created a need for methods and compositions which may be useful in the treatment diagnosis and prevention of malaria infections. The present invention meets these and other needs.

### **General Overview of Problems & Considerations in Directed Evolution**

The approach, termed directed evolution, of experimentally modifying a  
5 biological molecule towards a desirable property, can be achieved by mutagenizing  
one or more parental molecular templates and by identifying any desirable  
molecules among the progeny molecules. Currently available technologies in  
directed evolution include methods for achieving stochastic (i.e. random)  
mutagenesis and methods for achieving non-stochastic (non-random) mutagenesis.  
10 However, critical shortfalls in both types of methods are identified in the instant  
disclosure.

In prelude, it is noteworthy that it may be argued philosophically by some  
that all mutagenesis – if considered from an objective point of view – is non-  
15 stochastic; and furthermore that the entire universe is undergoing a process that – if  
considered from an objective point of view – is non-stochastic. Whether this is true  
is outside of the scope of the instant consideration. Accordingly, as used herein, the  
terms “randomness”, “uncertainty”, and “unpredictability” have subjective  
meanings, and the knowledge, particularly the predictive knowledge, of the designer  
20 of an experimental process is a determinant of whether the process is stochastic or  
non-stochastic.

By way of illustration, stochastic or random mutagenesis is exemplified by a  
situation in which a progenitor molecular template is mutated (modified or changed)  
25 to yield a set of progeny molecules having mutation(s) that are not predetermined.  
Thus, in an in vitro stochastic mutagenesis reaction, for example, there is not a  
particular predetermined product whose production is intended; rather there is an  
uncertainty – hence randomness – regarding the exact nature of the mutations  
achieved, and thus also regarding the products generated. In contrast, non-stochastic

or non-random mutagenesis is exemplified by a situation in which a progenitor molecular template is mutated (modified or changed) to yield a progeny molecule having one or more predetermined mutations. It is appreciated that the presence of background products in some quantity is a reality in many reactions where molecular processing occurs, and the presence of these background products does not detract from the non-stochastic nature of a mutagenesis process having a predetermined product.

Thus, as used herein, stochastic mutagenesis is manifested in processes such as error-prone PCR and stochastic shuffling, where the mutation(s) achieved are random or not predetermined. In contrast, as used herein, non-stochastic mutagenesis is manifested in instantly disclosed processes such as gene site-saturation mutagenesis and synthetic ligation reassembly, where the exact chemical structure(s) of the intended product(s) are predetermined.

In brief, existing mutagenesis methods that are non-stochastic have been serviceable in generating from one to only a very small number of predetermined mutations per method application, and thus produce per method application from one to only a few progeny molecules that have predetermined molecular structures. Moreover, the types of mutations currently available by the application of these non-stochastic methods are also limited, and thus so are the types of progeny mutant molecules.

In contrast, existing methods for mutagenesis that are stochastic in nature have been serviceable for generating somewhat larger numbers of mutations per method application – though in a random fashion & usually with a large but unavoidable contingency of undesirable background products. Thus, these existing stochastic methods can produce per method application larger numbers of progeny molecules, but that have undetermined molecular structures. The types of mutations

that can be achieved by application of these current stochastic methods are also limited, and thus so are the types of progeny mutant molecules.

It is instantly appreciated that there is a need for the development of non-stochastic mutagenesis methods that:

- 1) Can be used to generate large numbers of progeny molecules that have predetermined molecular structures;
- 2) Can be used to readily generate more types of mutations;
- 3) Can produce a correspondingly larger variety of progeny mutant molecules;
- 4) Produce decreased unwanted background products;
- 5) Can be used in a manner that is exhaustive of all possibilities; and
- 6) Can produce progeny molecules in a systematic & non-repetitive way.

15

The instant invention satisfies all of these needs.

**Directed Evolution Supplements Natural Evolution:** Natural evolution has been a springboard for directed or experimental evolution, serving both as a reservoir of methods to be mimicked and of molecular templates to be mutagenized. It is appreciated that, despite its intrinsic process-related limitations (in the types of favored &/or allowed mutagenesis processes) and in its speed, natural evolution has had the advantage of having been in process for millions of years & and throughout a wide diversity of environments. Accordingly, natural evolution (molecular mutagenesis and selection in nature) has resulted in the generation of a wealth of biological compounds that have shown usefulness in certain commercial applications.

20

25

However, it is instantly appreciated that many unmet commercial needs are discordant with any evolutionary pressure &/or direction that can be found in nature. Moreover, it is often the case that when commercially useful mutations would otherwise be favored at the molecular level in nature, natural evolution often  
5 overrides the positive selection of such mutations, e.g. when there is a concurrent detriment to an organism as a whole (such as when a favorable mutation is accompanied by a detrimental mutation). Additionally, natural evolution is often slow, and favors fidelity in many types of replication. Additionally still, natural evolution often favors a path paved mainly by consecutive beneficial mutations  
10 while tending to avoid a plurality of successive negative mutations, even though such negative mutations may prove beneficial when combined, or may lead - through a circuitous route - to final state that is beneficial.

Moreover, natural evolution advances through specific steps (e.g. specific  
15 mutagenesis and selection processes), with avoidance of less favored steps. For example, many nucleic acids do not reach close enough proximity to each other in a operative environment to undergo chimerization or incorporation or other types of transfers from one species to another. Thus, e.g., when sexual intercourse between 2 particular species is avoided in nature, the chimerization of nucleic acids from these  
20 2 species is likewise unlikely, with parasites common to the two species serving as an example of a very slow passageway for inter-molecular encounters and exchanges of DNA. For another example, the generation of a molecule causing self-toxicity or self-lethality or sexual sterility is avoided in nature. For yet another example, the propagation of a molecule having no particular immediate benefit to an organism is  
25 prone to vanish in subsequent generations of the organism. Furthermore, e.g., there is no selection pressure for improving the performance of molecule under conditions other than those to which it is exposed in its endogenous environment; e.g. a cytoplasmic molecule is not likely to acquire functional features extending beyond what is required of it in the cytoplasm. Furthermore still, the propagation of a

biological molecule is susceptible to any global detrimental effects – whether caused by itself or not – on its ecosystem. These and other characteristics greatly limit the types of mutations that can be propagated in nature.

5           On the other hand, directed (or experimental) evolution – particularly as provided herein – can be performed much more rapidly and can be directed in a more streamlined manner at evolving a predetermined molecular property that is commercially desirable where nature does not provide one &/or is not likely to provide. Moreover, the directed evolution invention provided herein can provide  
10 more wide-ranging possibilities in the types of steps that can be used in mutagenesis and selection processes. Accordingly, using templates harvested from nature, the instant directed evolution invention provides more wide-ranging possibilities in the types of progeny molecules that can be generated and in the speed at which they can be generated than often nature itself might be expected to in the same length of time.

15

          In a particular exemplification, the instantly disclosed directed evolution methods can be applied iteratively to produce a lineage of progeny molecules (e.g. comprising successive sets of progeny molecules) that would not likely be propagated (i.e., generated &/or selected for) in nature, but that could lead to the  
20 generation of a desirable downstream mutagenesis product that is not achievable by natural evolution.

#### **Previous Directed Evolution Methods Are Suboptimal:**

          Mutagenesis has been attempted in the past on many occasions, but by  
25 methods that are inadequate for the purpose of this invention. For example, previously described non-stochastic methods have been serviceable in the generation of only very small sets of progeny molecules (comprised often of merely a solitary progeny molecule). By way of illustration, a chimeric gene has been made by joining 2 polynucleotide fragments using compatible sticky ends generated by



restriction enzyme(s), where each fragment is derived from a separate progenitor (or parental) molecule. Another example might be the mutagenesis of a single codon position (i.e. to achieve a codon substitution, addition, or deletion) in a parental polynucleotide to generate a single progeny polynucleotide encoding for a single site-mutagenized polypeptide.

Previous non-stochastic approaches have only been serviceable in the generation of but one to a few mutations per method application. Thus, these previously described non-stochastic methods thus fail to address one of the central goals of this invention, namely the exhaustive and non-stochastic chimerization of nucleic acids. Accordingly previous non-stochastic methods leave untapped the vast majority of the possible point mutations, chimerizations, and combinations thereof, which may lead to the generation of highly desirable progeny molecules.

In contrast, stochastic methods have been used to achieve larger numbers of point mutations and/or chimerizations than non-stochastic methods; for this reason, stochastic methods have comprised the predominant approach for generating a set of progeny molecules that can be subjected to screening, and amongst which a desirable molecular species might hopefully be found. However, a major drawback of these approaches is that – because of their stochastic nature – there is a randomness to the exact components in each set of progeny molecules that is produced. Accordingly, the experimentalist typically has little or no idea what exact progeny molecular species are represented in a particular reaction vessel prior to their generation. Thus, when a stochastic procedure is repeated (e.g. in a continuation of a search for a desirable progeny molecule), the re-generation and re-screening of previously discarded undesirable molecular species becomes a labor-intensive obstruction to progress, causing a circuitous – if not circular – path to be taken. The drawbacks of such a highly suboptimal path can be addressed by subjecting a stochastically generated set of progeny molecules to a labor-incurring

process, such as sequencing, in order to identify their molecular structures, but even this is an incomplete remedy.

Moreover, current stochastic approaches are highly unsuitable for  
5 comprehensively or exhaustively generating all the molecular species within a particular grouping of mutations, for attributing functionality to specific structural groups in a template molecule (e.g. a specific single amino acid position or a sequence comprised of two or more amino acids positions), and for categorizing and comparing specific grouping of mutations. Accordingly, current stochastic  
10 approaches do not inherently enable the systematic elimination of unwanted mutagenesis results, and are, in sum, burdened by too many inherently shortcomings to be optimal for directed evolution.

In a non-limiting aspect, the instant invention addresses these problems by  
15 providing non-stochastic means for comprehensively and exhaustively generating all possible point mutations in a parental template. In another non-limiting aspect, the instant invention further provides means for exhaustively generating all possible chimerizations within a group of chimerizations. Thus, the aforementioned problems are solved by the instant invention.

20

Specific shortfalls in the technological landscape addressed by this invention include:

1) Site-directed mutagenesis technologies, such as sloppy or low-fidelity  
25 PCR, are ineffective for systematically achieving at each position (site) along a polypeptide sequence the full (saturated) range of possible mutations (i.e. all possible amino acid substitutions).

2) There is no relatively easy systematic means for rapidly analyzing the large amount of information that can be contained in a molecular sequence and in the

potentially colossal number or progeny molecules that could be conceivably obtained by the directed evolution of one or more molecular templates.

3) There is no relatively easy systematic means for providing comprehensive empirical information relating structure to function for molecular  
5 positions.

4) There is no easy systematic means for incorporating internal controls, such as positive controls, for key steps in certain mutagenesis (e.g. chimerization) procedures.

5) There is no easy systematic means to select for a specific group of  
10 progeny molecules, such as full-length chimeras, from among smaller partial sequences.

An exceedingly large number of possibilities exist for the purposeful and random combination of amino acids within a protein to produce useful hybrid  
15 proteins and their corresponding biological molecules encoding for these hybrid proteins, i.e., DNA, RNA. Accordingly, there is a need to produce and screen a wide variety of such hybrid proteins for a desirable utility, particularly widely varying random proteins.

20 The complexity of an active sequence of a biological macromolecule (e.g., polynucleotides, polypeptides, and molecules that are comprised of both polynucleotide and polypeptide sequences) has been called its information content ("IC"), which has been defined as the resistance of the active protein to amino acid  
25 sequence variation (calculated from the minimum number of invariable amino acids (bits) required to describe a family of related sequences with the same function). Proteins that are more sensitive to random mutagenesis have a high information content.

Molecular biology developments, such as molecular libraries, have allowed the identification of quite a large number of variable bases, and even provide ways to select functional sequences from random libraries. In such libraries, most residues can be varied (although typically not all at the same time) depending on compensating changes in the context. Thus, while a 100 amino acid protein can contain only 2,000 different mutations,  $20^{100}$  sequence combinations are possible.

Information density is the IC per unit length of a sequence. Active sites of enzymes tend to have a high information density. By contrast, flexible linkers of information in enzymes have a low information density.

Current methods in widespread use for creating alternative proteins in a library format are error-prone polymerase chain reactions and cassette mutagenesis, in which the specific region to be optimized is replaced with a synthetically mutagenized oligonucleotide. In both cases, a substantial number of mutant sites are generated around certain sites in the original sequence.

Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. In a mixture of fragments of unknown sequence, error-prone PCR can be used to mutagenize the mixture. The published error-prone PCR protocols suffer from a low processivity of the polymerase. Therefore, the protocol is unable to result in the random mutagenesis of an average-sized gene. This inability limits the practical application of error-prone PCR. Some computer simulations have suggested that point mutagenesis alone may often be too gradual to allow the large-scale block changes that are required for continued and dramatic sequence evolution. Further, the published error-prone PCR protocols do not allow for amplification of DNA fragments greater than 0.5 to 1.0 kb, limiting their practical application. In

addition, repeated cycles of error-prone PCR can lead to an accumulation of neutral mutations with undesired results, such as affecting a protein's immunogenicity but not its binding affinity.

5           In oligonucleotide-directed mutagenesis, a short sequence is replaced with a synthetically mutagenized oligonucleotide. This approach does not generate combinations of distant mutations and is thus not combinatorial. The limited library size relative to the vast sequence length means that many rounds of selection are unavoidable for protein optimization. Mutagenesis with synthetic  
10 oligonucleotides requires sequencing of individual clones after each selection round followed by grouping them into families, arbitrarily choosing a single family, and reducing it to a consensus motif. Such motif is re-synthesized and reinserted into a single gene followed by additional selection. This step process constitutes a statistical bottleneck, is labor intensive, and is not practical for many  
15 rounds of mutagenesis.

Error-prone PCR and oligonucleotide-directed mutagenesis are thus useful for single cycles of sequence fine tuning, but rapidly become too limiting when they are applied for multiple cycles.

20

Another limitation of error-prone PCR is that the rate of down-mutations grows with the information content of the sequence. As the information content, library size, and mutagenesis rate increase, the balance of down-mutations to up-mutations will statistically prevent the selection of further improvements  
25 (statistical ceiling).

In cassette mutagenesis, a sequence block of a single template is typically replaced by a (partially) randomized sequence. Therefore, the maximum information content that can be obtained is statistically limited by the number of

random sequences (i.e., library size). This eliminates other sequence families which are not currently best, but which may have greater long term potential.

Also, mutagenesis with synthetic oligonucleotides requires sequencing of  
5 individual clones after each selection round. Thus, such an approach is tedious and impractical for many rounds of mutagenesis.

Thus, error-prone PCR and cassette mutagenesis are best suited, and have been widely used, for fine-tuning areas of comparatively low information content.  
10 One apparent exception is the selection of an RNA ligase ribozyme from a random library using many rounds of amplification by error-prone PCR and selection.

In nature, the evolution of most organisms occurs by natural selection and  
15 sexual reproduction. Sexual reproduction ensures mixing and combining of the genes in the offspring of the selected individuals. During meiosis, homologous chromosomes from the parents line up with one another and cross-over part way along their length, thus randomly swapping genetic material. Such swapping or shuffling of the DNA allows organisms to evolve more rapidly.

20

In recombination, because the inserted sequences were of proven utility in a homologous environment, the inserted sequences are likely to still have substantial information content once they are inserted into the new sequence.

25 Theoretically there are 2,000 different single mutants of a 100 amino acid protein. However, a protein of 100 amino acids has  $20^{100}$  possible sequence combinations, a number which is too large to exhaustively explore by conventional methods. It would be advantageous to develop a system which

would allow generation and screening of all of these possible combination mutations.

Some workers in the art have utilized an *in vivo* site specific  
5 recombination system to generate hybrids of combine light chain antibody genes with heavy chain antibody genes for expression in a phage system. However, their system relies on specific sites of recombination and is limited accordingly. Simultaneous mutagenesis of antibody CDR regions in single chain antibodies (scFv) by overlapping extension and PCR have been reported.

10

Others have described a method for generating a large population of multiple hybrids using random *in vivo* recombination. This method requires the recombination of two different libraries of plasmids, each library having a different selectable marker. The method is limited to a finite number of  
15 recombinations equal to the number of selectable markers existing, and produces a concomitant linear increase in the number of marker genes linked to the selected sequence(s).

*In vivo* recombination between two homologous, but truncated, insect-  
20 toxin genes on a plasmid has been reported as a method of producing a hybrid gene. The *in vivo* recombination of substantially mismatched DNA sequences in a host cell having defective mismatch repair enzymes, resulting in hybrid molecule formation has been reported.

### 1.3. SUMMARY OF THE INVENTION

#### Directing an immune response so as to achieve an optimal response to vaccination.

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The present invention provides multicomponent genetic vaccines that include at least one, and preferably two or more genetic vaccine components that confer upon the vaccine the ability to direct an immune response so as to achieve an optimal response to vaccination. For example, the genetic vaccines can include  
10 a component that provides optimal antigen release; a component that provides optimal production of cytotoxic T lymphocytes; a component that directs release of an immunomodulator; a component that directs release of a chemokine; and/or a component that facilitates binding to, or entry into, a desired target cell type. For example, a component can confer improved improves binding to, and uptake of,  
15 the genetic vaccine to target cells such as antigen- expressing cells or antigen-presenting cells.

Additional components include those that direct antigen peptides derived from uptake of an antigen into a cell to presentation on either Class I or Class II  
20 molecules. For example, one can include a component that directs antigen peptides to presentation on Class I molecules and comprises a polynucleotide that encodes a protein such as tapasin, TAP-1 and TAP-2, and/or a component that directs antigen peptides to presentation on Class II molecules and comprises a polynucleotide that encodes a protein such as an endosomal or lysosomal  
25 protease.

In a particularly preferred aspect, this invention provides a method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized



modulatory effect on an immune response, the method comprising: creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; wherein optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation and iterative manner; whereby these directed evolution methods include the introduction of mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein; and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

In another particularly preferred aspect, this invention provides a method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized modulatory effect on an immune response, the method comprising: screening a library of non-stochastically generated progeny polynucleotides to identify an optimized non-stochastically generated progeny polynucleotide that has, or encodes a polypeptide that has, a modulatory effect on an immune response; wherein the optimized non-stochastically generated polynucleotide or the polypeptide encoded by the non-stochastically generated polynucleotide exhibits an enhanced ability to modulate an immune response compared to a parental polynucleotide from which the library was created.

In another particularly preferred aspect, this invention provides a method for obtaining an immunomodulatory polynucleotide that has an optimized modulatory effect on an immune response, or encodes a polypeptide that has an optimized modulatory effect on an immune response, the method comprising: a) creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; and b) screening the library to identify an

optimized non-stochastically generated progeny polynucleotide that has, or encodes a polypeptide that has, a modulatory effect on an immune response induced by a genetic vaccine vector; wherein the optimized non-stochastically generated polynucleotide or the polypeptide encoded by the non-stochastically generated polynucleotide exhibits an enhanced ability to modulate an immune response compared to a parental polynucleotide from which the library was created; whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation, and iterative manner; whereby these directed evolution methods include the introduction of point mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein; and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

15

In another particularly preferred aspect, this invention provides a method for obtaining an immunomodulatory polynucleotide that has, an optimized expression in a recombinant expression host, the method comprising: creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation and iterative manner; whereby these directed evolution methods include the introduction of mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein; and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as described herein.

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25

In another particularly preferred aspect, this invention provides a method for obtaining an immunomodulatory polynucleotide that has an optimized expression in a recombinant expression host, the method comprising: screening a library of non-stochastically generated progeny polynucleotides to identify an  
5 optimized non-stochastically generated progeny polynucleotide that has an optimized expression in a recombinant expression host when compared to the expression of a parental polynucleotide from which the library was created.

In another particularly preferred aspect, this invention provides a method  
10 for obtaining an immunomodulatory polynucleotide that has an optimized expression in a recombinant expression host, the method comprising: a)  
creating a library of non-stochastically generated progeny polynucleotides from a parental polynucleotide set; and b) screening a library of non-stochastically generated progeny polynucleotides to identify an optimized non-  
15 stochastically generated progeny polynucleotide that has an optimized expression in a recombinant expression host when compared to the expression of a parental polynucleotide from which the library was created; whereby optimization can thus be achieved using one or more of the directed evolution methods as described herein in any combination, permutation, and iterative manner; whereby these  
20 directed evolution methods include the introduction of point mutations by non-stochastic methods, including by "gene site saturation mutagenesis" as described herein; and whereby these directed evolution methods also include the introduction mutations by non-stochastic polynucleotide reassembly methods as described herein; including by synthetic ligation polynucleotide reassembly as  
25 described herein.

In one aspect, this invention provides that the ability to a vaccine, for example a genetic vaccine, or a component of a vaccine, for example a

component of a genetic vaccine by optimizing its immunogenicity. Moreover, the present invention provides for the modification of other properties, including its:

- Catalysed reaction(s)
- Reaction type
- 5   • Natural substrate(s)
- Substrate spectrum
- Product spectrum
- Inhibitor(s)
- Cofactor(s)/prosthetic group(s)
- 10   • Metal compounds/salts that affect it
- Turnover number
- Specific activity
- Km value
- pH optimum
- 15   • pH range
- Temperature optimum
- Temperature range

It is also instantly appreciated that the serviceability of a molecule with an immunogenic effect can be affected by additional physical properties, which can likewise be modified by directed evolution as provided herein, such as how it is affected by subjection to:

- Isolation/Preparation
- Purification
- 25   • Renaturing conditions (reversibility or retention of activity upon: heating and cooling, urea, salts, detergents, pH extremes)
- Crystallization
- pH
- Temperature

- Oxidation
- Organic solvent(s)
- Miscellaneous storage conditions

5

Moreover, the instant invention provides for the modification of molecule's immunogenic properties properties such as

- Exposure to biological compartments (stomach acids, in vivo degradation)
- Expression (e.g. Transcription &/or Translation) level
- 10 • mRNA stability
- Any in vivo interactions with other cells or biologicals

15 **Method for obtaining the genetic components.**

In some embodiments, one or more of the genetic vaccine components is obtained by a method that involves: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which can confer a desired property upon a genetic vaccine, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant nucleic acids; and (2) screening the library to identify at least one optimized recombinant component that exhibits an enhanced capacity to confer the desired property upon the genetic vaccine. If further optimization of the component is desired, the following additional steps can be conducted: (3) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least one optimized recombinant component with a further form of the nucleic acid, which is the same or different from the first and second forms, to produce a further library of recombinant

nucleic acids; (4) screening the further library to identify at least one further optimized recombinant component that exhibits an enhanced capacity to confer the desired property upon the genetic vaccine; and (5) repeating (3) and (4), as necessary, until the further optimized recombinant component exhibits a further enhanced capacity to confer the desired property upon the genetic vaccine.

### Members of a gene family

10 In some embodiments of the invention, the first form of the nucleic acid is a first member of a gene family and the second form of the nucleic acid comprises a second member of the gene family. Additional forms of the module nucleic acid can also be members of the gene family. As an example, the first member of the gene family can be obtained from a first species of organism and the second member of the gene family obtained from a second species of organism. If desired, the optimized recombinant genetic vaccine component obtained by the methods of the invention can be backcrossed by, for example, reassembling (&/or subjecting to one or more directed evolution methods described herein) the optimized recombinant genetic vaccine component with a molar excess of one or both of the first and second forms of the substrate nucleic acids to produce a further library of recombinant genetic vaccine components; and screening the further library to identify at least one optimized recombinant genetic vaccine component that further enhances the capability of a genetic vaccine vector that includes the component to modulate the immune response.

25

**Methods of obtaining a genetic vaccine component that confers upon a genetic vaccine vector an enhanced ability to replicate in a host cell.**

Additional embodiments of the invention provide methods of obtaining a  
5 genetic vaccine component that confers upon a genetic vaccine vector an  
enhanced ability to replicate in a host cell. These methods involve creating a  
library of recombinant nucleic acids by subjecting to reassembly (&/or one or  
more additional directed evolution methods described herein) at least two forms of  
a polynucleotide that can confer episomal replication upon a vector that contains  
10 the polynucleotide; introducing into a population of host cells a library of vectors,  
each of which contains a member of the library of recombinant nucleic acids and  
a polynucleotide that encodes a cell surface antigen; propagating the population of  
host cells for multiple generations; and identifying cells which display the cell  
surface antigen on a surface of the cell, wherein cells which display the cell  
15 surface antigen are likely to harbor a vector that contains a recombinant vector  
module which enhances the ability of the vector to replicate episomally.

**Obtaining genetic vaccine components that confer upon a vector an enhanced**  
20 **ability to replicate in a host cell.**

Genetic vaccine components that confer upon a vector an enhanced ability  
to replicate in a host cell can also be obtained by creating a library of recombinant  
nucleic acids by subjecting to reassembly (&/or one or more additional directed  
25 evolution methods described herein) at least two forms of a polynucleotide  
derived from a human papillomavirus that can confer episomal replication upon a  
vector that contains the polynucleotide; introducing a library of vectors, each of  
which contains a member of the library of recombinant nucleic acids, into a

population of host cells; propagating the host cells for a plurality of generations; and identifying cells that contain the vector.

5 In additional embodiments, the invention provides methods obtaining a genetic vaccine component that confers upon a vector an enhanced ability to replicate in a human host cell by creating a library of recombinant nucleic acids by subjecting to reassembly (&/or one or more additional directed evolution methods described herein) at least two forms of a polynucleotide that can confer episomal replication upon a vector that contains the polynucleotide; introducing a  
10 library of genetic vaccine vectors, each of which comprises a member of the library of recombinant nucleic acids, into a test system that mimics a human immune response; and determining whether the genetic vaccine vector replicates or induces an immune response in the test system. A suitable test system can involve human skin cells present as a xenotransplant on skin of an  
15 immunocompromised non-human host animal, for example, or a non-human mammal that comprises a functional human immune system. Replication in these systems can be detected by determining whether the animal exhibits an immune response against the antigen.

20 The invention also provides methods of obtaining a genetic vaccine component that confers upon a genetic vaccine an enhanced ability to enter an antigen-presenting cell. These methods involve creating a library of recombinant nucleic acids by subjecting to reassembly (&/or one or more additional directed evolution methods described herein) at least two forms of a polynucleotide that  
25 can confer episomal replication upon a vector that contains the polynucleotide; introducing a library of genetic vaccine vectors, each of which comprises a member of the library of recombinant nucleic acids, into a population of antigen-presenting or antigen-processing cells; and determining the percentage of cells in the population which contain the nucleic acid vector. Antigen-presenting or



antigen-processing cells of interest include, for example, B cells, monocytes/macrophages, dendritic cells, Langerhans cells, keratinocytes, and muscle cells.

5           The present invention provides methods of obtaining a polynucleotide that has a modulatory effect on an immune response that is induced by a genetic vaccine, either directly (i.e., as an immunomodulatory polynucleotide) or indirectly (i.e., upon translation of the polynucleotide to create an immunomodulatory polypeptide. The methods of the invention involve: creating a  
10 library of experimentally generated (in vitro &/or in vivo) polynucleotides; and screening the library to identify at least one optimized experimentally generated (in vitro &/or in vivo) polynucleotide that exhibits, either by itself or through the encoded polypeptide, an enhanced ability to modulate an immune response than a form of the nucleic acid from which the library was created. Examples include,  
15 for example, CpG-rich polynucleotide sequences, polynucleotide sequences that encode a costimulator (e.g., B7-1, B7-2, CD1, CD40, CD154 (ligand for CD40), CD150 (SLAM), or a cytokine. The screening step used in these methods can include, for example, introducing genetic vaccine vectors which comprise the library of recombinant nucleic acids into a cell, and identifying cells which  
20 exhibit an increased ability to modulate an immune response of interest or increased ability to express an immunomodulatory molecule. For example, a library of recombinant cytokine-encoding nucleic acids can be screened by testing the ability of cytokines encoded by the nucleic acids to activate cells which contain a receptor for the cytokine. The receptor for the cytokine can be native to  
25 the cell, or can be expressed from a heterologous nucleic acid that encodes the cytokine receptor. For example, the optimized costimulators can be tested to identify those for which the cells or culture medium are capable of inducing a predominantly  $T_H2$  immune response, or a predominantly  $T_H1$  immune response.

In some embodiments, the polynucleotide that has a modulatory effect on an immune response is obtained by: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid that is, or encodes a molecule that is, involved in modulating an immune response, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of experimentally generated (in vitro &/or in vivo) polynucleotides; and (2) screening the library to identify at least one optimized experimentally generated (in vitro &/or in vivo) polynucleotide that exhibits, either by itself or through the encoded polypeptide, an enhanced ability to modulate an immune response than a form of the nucleic acid from which the library was created. If additional optimization is desired, the method can further involve: (3) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least one optimized experimentally generated (in vitro &/or in vivo) polynucleotide with a further form of the nucleic acid, which is the same or different from the first and second forms, to produce a further library of experimentally generated (in vitro &/or in vivo) polynucleotides; (4) screening, the further library to identify at least one further optimized experimentally generated (in vitro &/or in vivo) polynucleotide that exhibits an enhanced ability to modulate an immune response than a form of the nucleic acid from which the library was created.; and (5) repeating (3) and (4), as necessary, until the further optimized experimentally generated (in vitro &/or in vivo) polynucleotide exhibits an further enhanced ability to modulate an immune response than a form of the nucleic acid from which the library was created.

In some embodiments of the invention, the library of experimentally generated (in vitro &/or in vivo) polynucleotides is screened by: expressing the experimentally generated (in vitro &/or in vivo) polynucleotides so that the encoded peptides or polypeptides are produced as fusions with a protein displayed on the surface of a replicable genetic package; contacting the replicable genetic

packages with a plurality of cells that display the receptor; and identifying cells that exhibit a modulation of an immune response mediated by the receptor.

The invention also provides methods for obtaining a polynucleotide that  
5 encodes an accessory molecule that improves the transport or presentation of  
antigens by a cell. These methods involve creating a library of experimentally  
generated (in vitro &/or in vivo) polynucleotides by subjecting to reassembly  
(&/or one or more additional directed evolution methods described herein) nucleic  
acids that encode all or part of the accessory molecule; and screening the library  
10 to identify an optimized experimentally generated (in vitro &/or in vivo)  
polynucleotide that encodes a recombinant accessory molecule that confers upon  
a cell an increased or decreased ability to transport or present an antigen on a  
surface of the cell compared to an accessory molecule encoded by the non-  
recombinant nucleic acids. In some embodiments, the screening step involves:  
15 introducing the library of experimentally generated (in vitro &/or in vivo)  
polynucleotides into a genetic vaccine vector that encodes an antigen to form a  
library of vectors; introducing the library of vectors into mammalian cells; and  
identifying mammalian cells that exhibit increased or decreased immunogenicity  
to the antigen.

20

In some embodiments of the invention, the cytokine that is optimized is  
interleukin-12 and the screening is performed by growing mammalian cells which  
contain the genetic vaccine vector in a culture medium, and detecting whether T  
cell proliferation or T cell differentiation is induced by contact with the culture  
25 medium. In another embodiment, the cytokine is interferon- and the screening is  
performed by expressing the recombinant vector module as a fusion protein which  
is displayed on the surface of a bacteriophage to form a phage display library, and  
identifying phage library members which are capable of inhibiting proliferation of  
a B cell line. Another embodiment utilizes B7-1 (CD80) or B7-2 (CD86) as the

costimulator and the cell or culture medium is tested for ability to modulate an immune response.

The invention provides methods of using stochastic (e.g. polynucleotide  
5 shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to obtain optimized recombinant vector modules that encode cytokines and other costimulators that exhibit reduced immunogenicity compared to a corresponding polypeptide encoded by a non-optimized vector module. The reduced  
immunogenicity can be detected by introducing a cytokine or costimulator  
10 encoded by the recombinant vector module into a mammal and determining whether an immune response is induced against the cytokine.

The invention also provides methods of obtaining optimized immunomodulatory sequences that encode a cytokine antagonist. For example,  
15 suitable cytokine agonists include a soluble cytokine receptor and a transmembrane cytokine receptor having, a defective signal sequence. Examples include sIL-10R and sIL- 4R, and the like.

The present invention provides methods for obtaining a cell-specific  
20 binding molecule that is useful for increasing uptake or specificity of a genetic vaccine to a target cell. The methods involve: creating a library of experimentally generated (in vitro &/or in vivo) polynucleotides that by reassembling (&/or subjecting to one or more directed evolution methods described herein) a nucleic acid that encodes a polypeptide that comprises a nucleic acid binding domain and  
25 a nucleic acid that encodes a polypeptide that comprises a cell-specific binding domain; and screening the library to identify a experimentally generated (in vitro &/or in vivo) polynucleotide that encodes a binding molecule that can bind to a nucleic acid and to a cell-specific receptor. Target cells of particular interest

include antigen-presenting and antigen-processing cells, such as muscle cells, monocytes, dendritic cells, B cells, Langerhans cells, keratinocytes, and M-cells.

In some embodiments, the methods of the invention for obtaining a cell-specific binding moiety useful for increasing uptake or specificity of a genetic vaccine to a target cell involve:

- (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprises a polynucleotide that encodes a nucleic acid binding domain and at least first and second forms of a nucleic acid which comprises a cell-specific ligand that specifically binds to a protein on the surface of a cell of interest, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant binding moiety-encoding nucleic acids;
- (2) transfecting into a population of host cells a library of vectors, each of which comprises: a) a binding site specific for the nucleic acid binding domain and b) a member of the library of recombinant binding moiety-encoding nucleic acids, wherein the recombinant binding moiety is expressed and binds to the binding site to form a vector-binding moiety complex;
- (3) lysing the host cells under conditions that do not disrupt binding of the vector-binding moiety complex;
- (4) contacting the vector-binding moiety complex with a target cell of interest; and

(5) identifying target cells that contain a vector and isolating the optimized recombinant cell-specific binding moiety nucleic acids from these target cells.

5           If further optimization is desired, the methods can further involve:

(6) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least one optimized recombinant binding moiety-encoding nucleic acid with a further form of the polynucleotide that encodes  
10           a nucleic acid binding domain and/or a further form of the polynucleotide that encodes a cell-specific ligand, which are the same or different from the first and second forms, to produce a further library of recombinant binding moiety-encoding nucleic acids;

15           (7) transfecting into a population of host cells a library of vectors that comprise: a) a binding site specific for the nucleic acid binding domain and 2) the recombinant binding moiety-encoding nucleic acids, wherein the recombinant binding moiety is expressed and binds to the binding site to form a vector- binding moiety complex;

20           (8) lysing the host cells under conditions that do not disrupt binding of the vector-binding moiety complex;

(9) contacting the vector-binding moiety complex with a target cell of interest  
25           and identifying target cells that contain the vector; and

(10) isolating the optimized recombinant binding moiety nucleic acids from the target cells which contain the vector; and

(11) repeating (6) through (10), as necessary, to obtain a further optimized cell-specific binding moiety useful for increasing uptake or specificity of a genetic vaccine vector to a target cell.

5           The invention also provides cell-specific recombinant binding moieties produced by expressing in a host cell an optimized recombinant binding moiety-encoding nucleic acid obtained by the methods of the invention.

          In another embodiment, the invention provides genetic vaccines that  
10   include:  
a) an optimized recombinant binding moiety that comprises a nucleic acid binding domain and a cell-specific ligand, and b) a polynucleotide sequence that comprises a binding site, wherein the nucleic acid binding domain is capable of specifically binding to the binding site.

15           A further embodiment of the invention provides methods for obtaining an optimized cell-specific binding moiety useful for increasing uptake, efficacy, or specificity of a genetic vaccine for a target cell by:

20   (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid that comprises a polynucleotide which encodes a non-toxic receptor binding moiety-of an enterotoxin or other toxin, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of  
25   recombinant nucleic acids;

(2) transfecting vectors that contain the library of nucleic acids into a population of host cells, wherein the nucleic acids are expressed to form recombinant cell- specific binding moiety polypeptides;

(3) contacting the recombinant cell-specific binding moiety polypeptides with a cell surface receptor of a target cell; and

- 5 (4) determining which recombinant cell-specific binding moiety polypeptides exhibit enhanced ability to bind to the target cell. Methods of enhancing uptake of a genetic vaccine vector by a target cell by coating the genetic vaccine vector with an optimized recombinant cell-specific binding moiety produced by these methods are also provided by the invention.

10

The present invention also provides methods for evolving a vaccine delivery vehicle, genetic vaccine vector, or a vector component to obtain an optimized delivery vehicle or component that has, or confers upon a vector, enhanced ability to enter a selected mammalian tissue upon administration to a  
15 mammal. These methods involve:

(1) reassembling (&/or subjecting to one or more directed evolution methods described herein) members of a pool of polynucleotides to produce a library of experimentally generated (in vitro &/or in vivo) polynucleotides;

20

(2) administering to a test animal a library of replicable genetic packages, each of which comprises a member of the library of experimentally generated (in vitro &/or in vivo) polynucleotides operably linked to a polynucleotide that encodes a display polypeptide, wherein the experimentally generated (in  
25 vitro &/or in vivo) polynucleotide and the display polypeptide are expressed as a fusion protein which is which is displayed on the surface of the replicable genetic package; and



(3) recovering replicable genetic packages that are present in the selected tissue of the test animal at a suitable time after administration, wherein recovered replicable genetic packages have enhanced ability to enter the selected mammalian tissue upon administration to the mammal.

5

If further optimization of the delivery vehicle is desired, the methods of the invention further involve:

(4) reassembling (&/or subjecting to one or more directed evolution methods described herein) a nucleic acid that comprises at least one experimentally generated (in vitro &/or in vivo) polynucleotide obtained from a replicable genetic package recovered from the selected tissue with a further pool of polynucleotides to produce a further library of experimentally generated (in vitro &/or in vivo) polynucleotides;

(5) administering to a test animal a library of replicable genetic packages, each of which comprises a member of the further library of experimentally generated (in vitro &/or in vivo) polynucleotides operably linked to a polynucleotide that encodes a display polypeptide, wherein the experimentally generated (in vitro &/or in vivo) polynucleotide and the display polypeptide are expressed as a fusion protein which is which is displayed on the surface of the replicable genetic package;

(6) recovering replicable genetic packages that are present in the selected tissue of the test animal at a suitable time after administration; and

(7) repeating (4) through (6), as necessary, to obtain a further optimized recombinant delivery vehicle that exhibits further enhanced ability to enter a selected mammalian tissue upon administration to a mammal. Methods of

administration that are of particular interest include, for example, oral, topical, and inhalation. Where the administration is intravenous, mammalian tissues of interest include, for example, lymph node and spleen.

5           In another embodiment, the invention provides methods for evolving a vaccine delivery vehicle, genetic vaccine vector, or a vector component to obtain an optimized delivery vehicle or component to obtain an optimized delivery vehicle or vector component that has, or confers upon a vector containing the component, enhanced specificity for antigen-presenting cells by:

10

(1) reassembling (&/or subjecting to one or more directed evolution methods described herein) members of a pool of polynucleotides to produce a library of experimentally generated (in vitro &/or in vivo) polynucleotides;

15

(2) producing a library of replicable genetic packages, each of which comprises a member of the library of experimentally generated (in vitro &/or in vivo) polynucleotides operably linked to a polynucleotide that encodes a display polypeptide, wherein the experimentally generated (in vitro &/or in vivo) polynucleotide and the display polypeptide are expressed as a fusion protein which is which is displayed on the surface of the replicable genetic package;

20

(3) contacting the library of recombinant replicable genetic packages with a non-APC to remove replicable genetic packages that display non-APC-specific fusion polypeptides; and

25

(4) contacting the recombinant replicable genetic packages that did not bind to the non-APC with an APC and recovering those that bind to the APC,

wherein the recovered replicable genetic packages are capable of specifically binding to APCs.

5 In an additional embodiment, the invention provides methods for evolving a vaccine delivery vehicle, genetic vaccine vector, or a vector component to obtain an optimized delivery vehicle or component to obtain an optimized delivery vehicle or vector component that has, or confers upon a vector containing the component, an enhanced ability to enter a target cell by:

- 10 (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which encodes an invasin polypeptide, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant invasin nucleic acids;
- 15 (2) producing a library of recombinant bacteriophage, each of which displays on the bacteriophage surface a fusion polypeptide encoded by a chimeric gene that comprises a recombinant invasin nucleic acid operably linked to a polynucleotide that encodes a display polypeptide;
- 20 (3) contacting the library of recombinant bacteriophage with a population of target cells;
- (4) removing unbound phage and phage which is bound to the surface of the target cells; and
- 25 (5) recovering phage which are present within the target cells, wherein the recovered phage are enriched for phage that have enhanced ability to enter the target cells.

In some embodiments, the optimized recombinant genetic vaccine vectors, delivery vehicles, or vector components obtained using these methods exhibit improved ability to enter an antigen presenting cell. These methods can involve  
5 washing the cells after the transfection step to remove vectors which did not enter an antigen presenting cell.; culturing the cells for a predetermined time after transfection; lysing the antigen presenting cells; and isolating the optimized recombinant genetic vaccine vector from the cell lysate.

10 **Antigen presenting cells that contain an optimized recombinant genetic vaccine vectors can be identified by, for example, detecting expression of a marker gene that is included in the vectors.**

The invention also provides methods of evolving a bacteriophage-derived  
15 vaccine delivery vehicle to obtain a delivery vehicle having enhanced ability to enter a target cell. These methods involve the steps of.

(1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which  
20 encodes an invasin polypeptide, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant invasin nucleic acids;

(2) producing a library of recombinant bacteriophage, each of which displays  
25 on the bacteriophage surface a fusion polypeptide encoded by a chimeric gene that comprises a recombinant invasin nucleic acid operably linked to a polynucleotide that encodes a display polypeptide;

- (3) contacting the library of recombinant bacteriophage with a population of target cells;
- 5 (4) removing unbound phage and phage which is bound to the surface of the target cells; and
- (5) recovering phage which are present within the target cells, wherein the recovered phage are enriched for phage that have enhanced ability to enter the target cells. Again, if further optimization is desired, the methods can
- 10 include the further steps of.
- (6) reassembling (&/or subjecting to one or more directed evolution methods described herein) a nucleic acid which comprises at least one recombinant invasin nucleic acid obtained from a bacteriophage which is recovered from
- 15 a target cell with a further pool of polynucleotides to produce a further library of recombinant invasin polynucleotides;
- (7) producing a further library of recombinant bacteriophage, each of which displays on the bacteriophage surface a fusion polypeptide encoded by a
- 20 chimeric gene that comprises a recombinant invasin nucleic acid operably linked to a polynucleotide that encodes a display polypeptide;
- (8) contacting the library of recombinant bacteriophage with a population of target cells;
- 25 (9) removing unbound phage and phage which is bound to the surface of the target cells; and
- (10) recovering phage which are present within the target cells; and

(11) repeating (6) through (10), as necessary, to obtain a further optimized recombinant delivery vehicle which exhibits further enhanced ability to enter the target cells.

5

In some embodiments the methods of evolving a bacteriophage-derived vaccine delivery vehicle to obtain a delivery vehicle having enhanced ability to enter a target cell can include the additional steps of.

- 10 (12) inserting into the optimized recombinant delivery vehicle a polynucleotide which encodes an antigen of interest, wherein the antigen of interest is expressed as a fusion polypeptide which comprises a second display polypeptide;
- 15 (13) administering the delivery vehicle to a test animal; and (14) determining whether the delivery vehicle is capable of inducing a CTL response in the test animal.

Alternatively, the following steps can be employed:

20

- (12) inserting into the optimized recombinant delivery vehicle a polynucleotide which encodes an antigen of interest, wherein the antigen of interest is expressed as a fusion polypeptide which comprises a second display polypeptide;

25

- (13) administering the delivery vehicle to a test animal; and
- (14) determining whether the delivery vehicle is capable of inducing neutralizing antibodies against a pathogen which comprises the antigen of

interest. An example of a target cell of interest for these methods is an antigen-presenting cell.

The present invention provides recombinant multivalent antigenic polypeptides that include a first antigenic determinant from a first disease-associated polypeptide and at least a second antigenic determinant from a second disease-associated polypeptide. The disease-associated polypeptides can be selected from the group consisting of cancer antigens, antigens associated with autoimmunity disorders, antigens associated with inflammatory conditions, antigens associated with allergic reactions, antigens associated with infectious agents, and other antigens that are associated with a disease condition.

In another embodiment, the invention provides a recombinant antigen library that contains recombinant nucleic acids that encode antigenic polypeptides. The libraries are typically obtained by reassembling (&/or subjecting to one or more directed evolution methods described herein), at least first and second forms of a nucleic acid which includes a polynucleotide sequence that encodes a disease-associated antigenic polypeptide, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant nucleic acids.

Another embodiment of the invention provides methods of obtaining a polynucleotide that encodes a recombinant antigen having improved ability to induce an immune response to a disease condition. These methods involve:

- (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprises a polynucleotide sequence that encodes an antigenic polypeptide that is associated with the disease condition, wherein the first and second

forms differ from each other in two or more nucleotides, to produce a library of recombinant nucleic acids; and

- 5 (2) screening the library to identify at least one optimized recombinant nucleic acid that encodes an optimized recombinant antigenic polypeptide that has improved ability to induce an immune response to the disease condition.

These methods optionally further involve:

- 10 (3) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least one optimized recombinant nucleic acid with a further form of the nucleic acid, which is the same or different from the first and second forms, to produce a further library of recombinant nucleic acids;
- 15 (4) screening the further library to identify at least one further optimized recombinant nucleic acid that encodes a polypeptide that has improved ability to induce an immune response to the disease condition; and
- 20 (5) repeating (3) and (4), as necessary, until the further optimized recombinant nucleic acid encodes a polypeptide that has improved ability to induce an immune response to the disease condition.

In some embodiments, the optimized recombinant nucleic acid encodes a multivalent antigenic polypeptide and the screening is accomplished by

25 expressing the library of recombinant nucleic acids in a phage display expression vector such that the recombinant antigen is expressed as a fusion protein with a phage polypeptide that is displayed on a phage particle surface; contacting the phage with a first antibody that is specific for a first serotype of the pathogenic agent and selecting those phage that bind to the first antibody; and contacting



those phage that bind to the first antibody with a second antibody that is specific for a second serotype of the pathogenic agent and selecting those phage that bind to the second antibody; wherein those phage that bind to the first antibody and the second antibody express a multivalent antigenic polypeptide.

5

**The invention also provides methods of obtaining a recombinant viral vector which has an enhanced ability to induce an antiviral response in a cell.**

10 **Methods of obtaining a recombinant genetic vaccine component that confers upon a genetic vaccine an enhanced ability to induce a desired immune response in a mammal**

In additional embodiments, the invention provides methods of obtaining a  
15 recombinant genetic vaccine component that confers upon a genetic vaccine an enhanced ability to induce a desired immune response in a mammal. These methods involve: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprise a genetic vaccine vector, wherein the first and second forms  
20 differ from each other in two or more nucleotides, to produce a library of recombinant genetic vaccine vectors; (2) transfecting the library of recombinant vaccine vectors into a population of mammalian cells selected from the group consisting of peripheral blood T cells, T cell clones, freshly isolated monocytes/macrophages and dendritic cells; (3) staining the cells for the presence  
25 of one or more cytokines and identifying cells which exhibit a cytokine staining pattern indicative of the desired immune response; and (4) obtaining recombinant vaccine vector nucleic acid sequences from the cells which exhibit the desired cytokine staining pattern.

**Methods of improving the ability of a genetic vaccine vector to modulate an immune response.**

5           Also provided by the invention are methods of improving the ability of a genetic vaccine vector to modulate an immune response by: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprise a genetic vaccine vector, wherein the first and second forms differ from each other in two or more  
10   nucleotides, to produce a library of recombinant genetic vaccine vectors; (2) transfecting the library of recombinant genetic vaccine vectors into a population of antigen presenting cells; and (3) isolating from the cells optimized recombinant genetic vaccine vectors which exhibit enhanced ability to modulate a desired  
15   immune response.

**Methods of obtaining a recombinant genetic vaccine vector that has an enhanced ability to induce a desired immune response in a mammal upon administration to the skin of the mammal.**

20           Another embodiment of the invention provides methods of obtaining a recombinant genetic vaccine vector that has an enhanced ability to induce a desired immune response in a mammal upon administration to the skin of the mammal. These methods involve: (1) reassembling (&/or subjecting to one or  
25   more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprise a genetic vaccine vector, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant genetic vaccine vectors; (2) topically applying the library of recombinant genetic vaccine vectors to skin of a mammal; (3) identifying

vectors that induce an immune response; and (4) recovering genetic vaccine vectors from the skin cells which contain vectors that induce an immune response.

- 5    **Methods of inducing an immune response in a mammal by topically applying to skin of the mammal a genetic vaccine vector, wherein the genetic vaccine vector is optimized for topical application through use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly.**

10

          The invention also provides methods of inducing an immune response in a mammal by topically applying to skin of the mammal a genetic vaccine vector, wherein the genetic vaccine vector is optimized for topical application through use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly. In some embodiments, the genetic vaccine is administered as a formulation selected from the group consisting of a transdermal patch, a cream, naked DNA, a mixture of DNA and a transfection-enhancing agent. Suitable transfection-enhancing agents include one or more agents selected from the group consisting of a lipid, a liposome, a protease, and a lipase.

20

          Alternatively, or in addition, the genetic vaccine can be administered after pretreatment of the skin by abrasion or hair removal.

**Methods of obtaining an optimized genetic vaccine component that confers upon a genetic vaccine containing the component an enhanced ability to induce or inhibit apoptosis of a cell into which the vaccine is introduced.**

5           In another embodiment, the invention provides methods of obtaining an optimized genetic vaccine component that confers upon a genetic vaccine containing the component an enhanced ability to induce or inhibit apoptosis of a cell into which the vaccine is introduced. These methods involve: (1) reassembling (&/or subjecting to one or more directed evolution methods  
10 described herein) at least first and second forms of a nucleic acid which comprise a nucleic acid that encodes an apoptosis- modulating polypeptide, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant nucleic acids; (2) transfecting the library of recombinant nucleic acids into a population of mammalian cells; (3) staining the  
15 cells for the presence of a cell membrane change which is indicative of apoptosis initiation; and (4) obtaining recombinant apoptosis-modulating genetic vaccine components from the cells which exhibit the desired apoptotic membrane changes.

20

**Methods of obtaining a genetic vaccine component that confers upon a genetic vaccine reduced susceptibility to a CTL immune response in a host mammal.**

25           Other embodiments of the invention provide methods of obtaining a genetic vaccine component that confers upon a genetic vaccine reduced susceptibility to a CTL immune response in a host mammal. These methods can involve: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which

comprises a gene that encodes an inhibitor of a CTL immune response, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant CTL inhibitor nucleic acids; (2) introducing genetic vaccine vectors which comprise the library of recombinant CTL inhibitor  
5 nucleic acids into a plurality of human cells; (3) selecting cells which exhibit reduced MHC class I molecule expression; and (4) obtaining optimized recombinant CTL inhibitor nucleic acids from the selected cells.

10 **Methods of obtaining a genetic vaccine component that confers upon a genetic vaccine reduced susceptibility to a CTL immune response in a host mammal.**

The invention also provides methods of obtaining a genetic vaccine  
15 component that confers upon a genetic vaccine reduced susceptibility to a CTL immune response in a host mammal. These methods involve: (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprises a gene that encodes an inhibitor of a CTL immune response, wherein the first and second forms differ  
20 from each other in two or more nucleotides, to produce a library of recombinant CTL inhibitor nucleic acids; (2) introducing viral vectors which comprise the library of recombinant CTL inhibitor nucleic acids into mammalian cells; (3) identifying mammalian cells which express a marker gene included in the viral vectors a predetermined time after introduction, wherein the identified cells are  
25 resistant to a CTL response; and (4) recovering as the genetic vaccine component the recombinant CTL inhibitor nucleic acids from the identified cells.

It is a general object of the invention to provide proteins and polypeptides that are derived from PfEMP1 proteins, nucleic acids encoding these proteins and

antibodies that are specifically immunoreactive with these proteins. It is a further object to provide methods of using these various compositions in diagnosis, treatment or prevention of the onset of symptoms of a malaria parasite infection. It is a further object to provide methods of screening compounds to identify  
5 further compositions which may be used in these methods.

In one embodiment, the present invention provides substantially pure polypeptides which have amino acid sequences substantially homologous to the amino acid sequence of a PfEMP1 protein, or biologically active fragments  
10 thereof.

In preferred aspects, the polypeptides of the present invention are substantially homologous to the amino acid sequence shown, described &/or referenced herein (including incorporated by reference), biologically active  
15 fragments or analogues thereof. Also provided are pharmaceutical compositions comprising these polypeptides.

In another embodiment, the present invention provides nucleic acids which encode the above-described polypeptides. Particularly preferred nucleic  
20 acids will be substantially homologous to a part or whole of the nucleic acid sequence shown, described &/or referenced herein (including incorporated by reference) or the nucleic acid encoding for the sequences shown, described &/or referenced herein (including incorporated by reference). The present invention also provides expression vectors comprising these nucleic acid sequences and  
25 cells capable of expressing same.

In an additional embodiment, the present invention provides antibodies which recognize and bind PfEMP1 polypeptides or biologically active fragments

thereof. More preferred are those peptides which recognize and bind PfEMP1 proteins associated with infection by more than one variant of *P. falciparum*.

In a further embodiment, the present invention provides methods of  
5 inhibiting the formation of PfEMP1/ligand complex, comprising contacting PfEMP1 or its ligands with polypeptides of the present invention.

In a related embodiment, the present invention provides methods of  
inhibiting sequestration of erythrocytes in a patient suffering from a malaria  
10 infection, comprising administering to said patient, an effective amount of a polypeptide of the present invention. such administration may be carried out prior to or following infection.

In still another embodiment, the present invention provides a method of  
15 detecting the presence or absence of PfEMP1 in a sample. The method comprises exposing the sample to an antibody of the invention, and detecting binding, if any, between the antibody and a component of the sample.

In an additional embodiment, the present invention provides a method of  
20 determining whether a test compound is an antagonist of PfEMP1/ligand complex formation. The method comprises incubating the test compound with PfEMP1 or a biologically active fragment thereof, and its ligand, under conditions which permit the formation of the complex. The amount of complex formed in the presence of the test compound is determined and compared with the amount of  
25 complex formed in the absence of the test compound. A decrease in the amount of complex formed in the presence of the test compound is indicative that the compound is an antagonist of PfEMP1/ligand complex formation.

### Summary of Directed Evolution Approaches

5 This invention also relates generally to the field of nucleic acid engineering and correspondingly encoded recombinant protein engineering. More particularly, the invention relates to the directed evolution of nucleic acids and screening of clones containing the evolved nucleic acids for resultant activity(ies) of interest, such nucleic acid activity(ies) &/or specified protein, particularly enzyme, activity(ies) of interest.

10 Mutagenized molecules provided by this invention may have chimeric molecules and molecules with point mutations, including biological molecules that contain a carbohydrate, a lipid, a nucleic acid, &/or a protein component, and specific but non-limiting examples of these include antibiotics, antibodies, enzymes, and steroidal and non-steroidal hormones.

15 This invention relates generally to a method of: 1) preparing a progeny generation of molecule(s) (including a molecule that is comprised of a polynucleotide sequence, a molecule that is comprised of a polypeptide sequence, and a molecules that is comprised in part of a polynucleotide sequence and in part of a polypeptide  
20 sequence), that is mutagenized to achieve at least one point mutation, addition, deletion, &/or chimerization, from one or more ancestral or parental generation template(s); 2) screening the progeny generation molecule(s) - preferably using a high throughput method - for at least one property of interest (such as an improvement in an enzyme activity or an increase in stability or a novel chemotherapeutic effect); 3)  
25 optionally obtaining &/or cataloguing structural &/or and functional information regarding the parental &/or progeny generation molecules; and 4) optionally repeating any of steps 1) to 3).



In a preferred embodiment, there is generated (e.g. from a parent polynucleotide template) - in what is termed "codon site-saturation mutagenesis" - a progeny generation of polynucleotides, each having at least one set of up to three contiguous point mutations (i.e. different bases comprising a new codon),  
5 such that every codon (or every family of degenerate codons encoding the same amino acid) is represented at each codon position. Corresponding to - and encoded by - this progeny generation of polynucleotides, there is also generated a set of progeny polypeptides, each having at least one single amino acid point mutation. In a preferred aspect, there is generated - in what is termed "amino acid  
10 site-saturation mutagenesis" - one such mutant polypeptide for each of the 19 naturally encoded polypeptide-forming alpha-amino acid substitutions at each and every amino acid position along the polypeptide. This yields - for each and every amino acid position along the parental polypeptide - a total of 20 distinct progeny polypeptides including the original amino acid, or potentially more than 21  
15 distinct progeny polypeptides if additional amino acids are used either instead of or in addition to the 20 naturally encoded amino acids

Thus, in another aspect, this approach is also serviceable for generating mutants containing - in addition to &/or in combination with the 20 naturally  
20 encoded polypeptide-forming alpha-amino acids - other rare &/or not naturally-encoded amino acids and amino acid derivatives. In yet another aspect, this approach is also serviceable for generating mutants by the use of - in addition to &/or in combination with natural or unaltered codon recognition systems of suitable hosts - altered, mutagenized, &/or designer codon recognition systems  
25 (such as in a host cell with one or more altered tRNA molecules).

In yet another aspect, this invention relates to recombination and more specifically to a method for preparing polynucleotides encoding a polypeptide by a method of *in vivo* re-assortment of polynucleotide sequences containing regions of

partial homology, assembling the polynucleotides to form at least one polynucleotide and screening the polynucleotides for the production of polypeptide(s) having a useful property.

5           In yet another preferred embodiment, this invention is serviceable for analyzing and cataloguing with respect to any molecular property (e.g. an enzymatic activity) or combination of properties allowed by current technology - the effects of any mutational change achieved (including particularly saturation mutagenesis). Thus, a comprehensive method is provided for determining the effect of changing each  
10 amino acid in a parental polypeptide into each of at least 19 possible substitutions. This allows each amino acid in a parental polypeptide to be characterized and catalogued according to its spectrum of potential effects on a measurable property of the polypeptide.

15           In another aspect, the method of the present invention utilizes the natural property of cells to recombine molecules and/or to mediate reductive processes that reduce the complexity of sequences and extent of repeated or consecutive sequences possessing regions of homology.

20           It is an object of the present invention to provide a method for generating hybrid polynucleotides encoding biologically active hybrid polypeptides with enhanced activities. In accomplishing these and other objects, there has been provided, in accordance with one aspect of the invention, a method for introducing polynucleotides into a suitable host cell and growing the host cell  
25 under conditions that produce a hybrid polynucleotide.

In another aspect of the invention, the invention provides a method for screening for biologically active hybrid polypeptides encoded by hybrid

polynucleotides. The present method allows for the identification of biologically active hybrid polypeptides with enhanced biological activities.

5

#### 1.4. BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1. Exonuclease Activity.** Figure 1 shows the activity of the enzyme exonuclease III. This is an exemplary enzyme that can be used to shuffle,  
10 assemble, reassemble, recombine, and/or concatenate polynucleotide building blocks. The asterisk indicates that the enzyme acts from the 3' direction towards the 5' direction of the polynucleotide substrate.

**Figure 2. Generation of A Nucleic Acid Building Block by Polymerase-Based**  
15 **Amplification.** Figure 2 illustrates a method of generating a double-stranded nucleic acid building block with two overhangs using a polymerase-based amplification reaction (e.g., PCR). As illustrated, a first polymerase-based amplification reaction using a first set of primers, F<sub>2</sub> and R<sub>1</sub>, is used to generate a blunt-ended product (labeled Reaction 1, Product 1), which is essentially identical  
20 to Product A. A second polymerase-based amplification reaction using a second set of primers, F<sub>1</sub> and R<sub>2</sub>, is used to generate a blunt-ended product (labeled Reaction 2, Product 2), which is essentially identical to Product B. These two products are then mixed and allowed to melt and anneal, generating a potentially useful double-stranded nucleic acid building block with two overhangs. In the  
25 example of Fig. 1, the product with the 3' overhangs (Product C) is selected for by nuclease-based degradation of the other 3 products using a 3' acting exonuclease, such as exonuclease III. Alternate primers are shown in parenthesis to illustrate serviceable primers may overlap, and additionally that serviceable primers may be of different lengths, as shown.

**Figure 3. Unique Overhangs And Unique Couplings.** Figure 3 illustrates the point that the number of unique overhangs of each size (e.g. the total number of unique overhangs composed of 1 or 2 or 3, etc. nucleotides) exceeds the number of unique couplings that can result from the use of all the unique overhangs of that size. For example, there are 4 unique 3' overhangs composed of a single nucleotide, and 4 unique 5' overhangs composed of a single nucleotide. Yet the total number of unique couplings that can be made using all the 8 unique single-nucleotide 3' overhangs and single-nucleotide 5' overhangs is 4.

10

**Figure 4. Unique Overall Assembly Order Achieved by Sequentially Coupling the Building Blocks**

Figure 4 illustrates the fact that in order to assemble a total of "n" nucleic acid building blocks, "n-1" couplings are needed. Yet it is sometimes the case that the number of unique couplings available for use is fewer than the "n-1" value. Under these, and other, circumstances a stringent non-stochastic overall assembly order can still be achieved by performing the assembly process in sequential steps. In this example, 2 sequential steps are used to achieve a designed overall assembly order for five nucleic acid building blocks. In this illustration the designed overall assembly order for the five nucleic acid building blocks is: 5'-(#1-#2-#3-#4-#5)-3', where #1 represents building block number 1, etc.

**Figure 5. Unique Couplings Available Using a Two-Nucleotide 3' Overhang.** Figure 5 further illustrates the point that the number of unique overhangs of each size (here, e.g. the total number of unique overhangs composed of 2 nucleotides) exceeds the number of unique couplings that can result from the use of all the unique overhangs of that size. For example, there are 16 unique 3' overhangs composed of two nucleotides, and another 16 unique 5' overhangs composed of two nucleotides, for a total of 32 as shown. Yet the total number of couplings that

are unique and not self-binding that can be made using all the 32 unique double-nucleotide 3' overhangs and double-nucleotide 5' overhangs is 12. Some apparently unique couplings have "identical twins" (marked in the same shading), which are visually obvious in this illustration. Still other overhangs contain  
5 nucleotide sequences that can self-bind in a palindromic fashion, as shown and labeled in this figure; thus they not contribute the high stringency to the overall assembly order.

**Figure 6. Generation of an Exhaustive Set of Chimeric Combinations by Synthetic Ligation Reassembly.** Figure 6 showcases the power of this invention in its ability to generate exhaustively and systematically all possible combinations of the nucleic acid building blocks designed in this example. Particularly large sets (or libraries) of progeny chimeric molecules can be generated. Because this method can be performed exhaustively and systematically, the method application  
15 can be repeated by choosing new demarcation points and with correspondingly newly designed nucleic acid building blocks, bypassing the burden of re-generating and re-screening previously examined and rejected molecular species. It is appreciated that, codon wobble can be used to advantage to increase the frequency of a demarcation point. In other words, a particular base can often be  
20 substituted into a nucleic acid building block without altering the amino acid encoded by progenitor codon (that is now altered codon) because of codon degeneracy. As illustrated, demarcation points are chosen upon alignment of 8 progenitor templates. Nucleic acid building blocks including their overhangs (which are serviceable for the formation of ordered couplings) are then designed  
25 and synthesized. In this instance, 18 nucleic acid building blocks are generated based on the sequence of each of the 8 progenitor templates, for a total of 144 nucleic acid building blocks (or double-stranded oligos). Performing the ligation synthesis procedure will then produce a library of progeny molecules comprised of yield of  $8^{18}$  (or over  $1.8 \times 10^{16}$ ) chimeras.

**Figure 7. Synthetic genes from oligos:.** According to one embodiment of this invention, double-stranded nucleic acid building blocks are designed by aligning a plurality of progenitor nucleic acid templates. Preferably these templates contain  
5 some homology and some heterology. The nucleic acids may encode related proteins, such as related enzymes, which relationship may be based on function or structure or both. Figure 7 shows the alignment of three polynucleotide progenitor templates and the selection of demarcation points (boxed) shared by all the progenitor molecules. In this particular example, the nucleic acid building  
10 blocks derived from each of the progenitor templates were chosen to be approximately 30 to 50 nucleotides in length.

**Figure 8. Nucleic acid building blocks for synthetic ligation gene reassembly.** Figure 8 shows the nucleic acid building blocks from the example in Figure 7.  
15 The nucleic acid building blocks are shown here in generic cartoon form, with their compatible overhangs, including both 5' and 3' overhangs. There are 22 total nucleic acid building blocks derived from each of the 3 progenitor templates. Thus, the ligation synthesis procedure can produce a library of progeny molecules comprised of yield of  $3^{22}$  (or over  $3.1 \times 10^{10}$ ) chimeras.

20

**Figure 9. Addition of Introns by Synthetic Ligation Reassembly.** Figure 9 shows in generic cartoon form that an intron may be introduced into a chimeric progeny molecule by way of a nucleic acid building block. It is appreciated that introns often have consensus sequences at both termini in order to render them  
25 operational. It is also appreciated that, in addition to enabling gene splicing, introns may serve an additional purpose by providing sites of homology to other nucleic acids to enable homologous recombination. For this purpose, and potentially others, it may be sometimes desirable to generate a large nucleic acid building block for introducing an intron. If the size is overly large easily

generating by direct chemical synthesis of two single stranded oligos, such a specialized nucleic acid building block may also be generated by direct chemical synthesis of more than two single stranded oligos or by using a polymerase-based amplification reaction as shown, described &/or referenced herein (including  
5 incorporated by reference).

**Figure 10. Ligation Reassembly Using Fewer Than All The Nucleotides Of An Overhang.** Figure 10 shows that coupling can occur in a manner that does not make use of every nucleotide in a participating overhang. The coupling is  
10 particularly lively to survive (e.g. in a transformed host) if the coupling reinforced by treatment with a ligase enzyme to form what may be referred to as a "gap ligation" or a "gapped ligation". It is appreciated that, as shown, this type of coupling can contribute to generation of unwanted background product(s), but it can also be used advantageously increase the diversity of the progeny library  
15 generated by the designed ligation reassembly.

**Figure 11. Avoidance of unwanted self-ligation in palindromic couplings.** As mentioned before and shown, described &/or referenced herein (including incorporated by reference), certain overhangs are able to undergo self-coupling to  
20 form a palindromic coupling. A coupling is strengthened substantially if it is reinforced by treatment with a ligase enzyme. Accordingly, it is appreciated that the lack of 5' phosphates on these overhangs, as shown, can be used advantageously to prevent this type of palindromic self-ligation. Accordingly, this invention provides that nucleic acid building blocks can be chemically made  
25 (or ordered) that lack a 5' phosphate group (or alternatively they can be remove – e.g. by treatment with a phosphatase enzyme such as a calf intestinal alkaline phosphatase (CIAP) – in order to prevent palindromic self-ligations in ligation reassembly processes.

**Figure 12. Site-directed mutagenesis by polymerase-based extension. Panel A.** This figure shows one method of site-directed mutagenesis, among many methods of site-directed mutagenesis, that are serviceable for performing site-saturation mutagenesis. **Section (1)** shows the first and second mutagenic primer annealed to a circular closed double-stranded plasmid. The dot and the open-sided triangle indicate the mutagenic sites in the mutagenic primers. The arrows indicate the direction of synthesis. **Section (2)** shows the newly synthesized (mutagenized) DNA strands annealed to each other. The parental DNA can be treated with a selection enzyme. The mutagenized DNA strands are shown as being annealed to form a double-stranded mutagenized circular DNA intermediate. The dot and the open-sided triangle indicate the mutagenic sites in the experimentally generated progeny (mutagenized) DNA strands. Note that the staggered openings on the mutagenized DNA strands form "sticky" ends. **Section (3)** shows the first and second mutagenic primer annealed to the mutagenized DNA strands of **Section (2)**. The arrows indicate the direction of synthesis. Note the opening on each of the mutagenized DNA strands (i.e. they have not been ligated). **Section (4)** shows a "Gapped Product", which is composed of second generation mutagenized DNA strands, synthesized using the mutagenized DNA strands (shown in Step (2)) as a template. The DNA strands of the "Gapped Product" are shown as being annealed to form a double-stranded mutagenized circular DNA intermediate. The dot and the open-sided triangle indicate the mutagenic sites in the mutagenized DNA strands. Note the large gap in each of the mutagenized DNA strands. **Section (5)** shows the "Gapped Product" annealed to the parental (non-mutated) plasmid, enabling polymerase-based synthesis to occur. The arrows indicate the direction of synthesis. **Section (6)** shows the newly synthesized DNA strands, as being annealed to form a double-stranded mutagenized circular DNA product. The dot and the open-sided triangle indicate the mutagenic sites in the mutagenized DNA strands. Note the staggered



openings on the mutagenized DNA strands. Also note the presence of both mutagenic sites on each of the mutagenized DNA strands.

**Panel B.** This figure shows two possible molecular structures produced from the amplification steps of Figure 12A. **Molecule (A)** is shown also in **Section (2)** of Figure 12A. **Molecule (B)** is also shown in **Section (6)** of Figure 12A.

**Figure 13. Site-directed mutagenesis by polymerase-based extension and ligase-based ligation. Panel A.** This figure shows one method of site-directed mutagenesis, among many methods of site-directed mutagenesis, that are serviceable for performing site-saturation mutagenesis. **Section (1)** shows the first and second mutagenic primer annealed to a circular closed double-stranded plasmid. The dot and the open-sided triangle indicate the mutagenic sites in the mutagenic primers. The arrows indicate the direction of synthesis. **Section (2)** shows the newly synthesized (mutagenized) DNA strands annealed to each other. The parental DNA can be treated with a selection enzyme. The mutagenized DNA strands are shown as being annealed to form a double-stranded mutagenized circular DNA intermediate. The dot and the open-sided triangle indicate the mutagenic sites in the experimentally generated progeny (mutagenized) DNA strands. Note that the staggered openings on the mutagenized DNA strands form "sticky" ends. **Section (3)** shows the resultant double-stranded mutagenized circular DNA molecule produced after the double-stranded mutagenized circular DNA intermediate of **Section (2)** is ligated (e.g. with T4 DNA ligase). **Section (4)** shows the first and second mutagenic primer annealed to the mutagenized DNA strands of **Section (3)**. The arrows indicate the direction of synthesis. **Section (5)** shows the recently generated (blue) mutagenized DNA strands as being annealed to form a double-stranded mutagenized circular DNA intermediate. The dot and the open-sided triangle indicate the mutagenic sites in the recently generated mutagenized DNA strands (blue). Note that the staggered

openings on the mutagenized DNA strands form "sticky ends". Also note the presence of both mutagenic sites on each of the two recently generated mutagenized DNA strands (blue). Note the opening on each of the mutagenized DNA strands (i.e. they have not been ligated). **Section (6)** shows the resultant double-stranded mutagenized circular DNA molecule produced after the double-stranded mutagenized circular DNA intermediate of **Section (5)** is ligated (e.g. using T4 DNA ligase). The dot and the open-sided triangle indicate the mutagenic sites in the mutagenized DNA molecules. Again, note the presence of both mutagenic sites on each of the mutagenized DNA strands.

10

**Panel B.** This figure shows two molecular structures produced from the amplification steps of Figure 13A. **Molecule (A)** is also shown in **Section (3)** of Figure 13A. **Molecule (B)** is produced in **Section (6)** of Figure 13A.

15

**Figure 14: Strategy for Obtaining and Using Nucleic Acid Binding Proteins that Facilitate Entry of Genetic Vaccines.**

Shown here is a strategy for obtaining and using nucleic acid binding proteins that facilitate entry of genetic vaccines, in particular, naked DNA, into target cells.

20 Members of a library obtained by the directed evolution methods described herein are linked to a coding region of M 13 protein VIII so that a fusion protein is displayed on the surface of the phage particles. Phage that efficiently enter the desired target tissue are identified, and the fusion protein is then used to coat a genetic vaccine nucleic acid.

**Figure 15: A schematic representation of a method for generating a chimeric, multivalent antigen that has immunogenic regions from multiple antigens.**

Antibodies to each of the non-chimeric parental immunogenic polypeptides are specific for the respective organisms (A, B, C). After carrying out the directed evolution and selection methods of the invention, however, a chimeric immunogenic polypeptide is obtained that is recognized by antibodies raised against each of the three parental immunogenic polypeptides.

**10 Figure 16A and Figure 16B: Method for Obtaining Non-Stochastically Generated Polypeptides that can induce a Broad-Spectrum Immune Response.**

Shown here is a schematic for a method by which one can obtain non-stochastically generated polypeptides that can induce a broad-spectrum immune response. In Figure 16A, wild-type immunogenic polypeptides from the pathogens A, B, and C provide protection against the corresponding pathogen from which the polypeptide is derived, but little or no cross-protection against the other pathogens (left panel). After evolving, an A/B/C chimeric polypeptide is obtained that can induce a protective immune response against all three pathogen types (right panel). In Figure 16B, directed evolution is used with substrate nucleic acids from two pathogen strains (A, B), which encode polypeptides that are protective only against the corresponding pathogen. After directed evolution, the resulting chimeric polypeptide can induce an immune response that is effective against not only the two parental pathogen strains, but also against a third strain of pathogen (C).

**Figure 17: Possible factors for determining whether a particular polynucleotide encodes an immunogenic polypeptide having a desired property.**

Shown here are some of the possible factors that can determine whether a particular polynucleotide encodes an immunogenic polypeptide having a desired property, such as enhanced immunogenicity and/or cross-reactivity. Those sequence regions that positively affect a particular property are indicated as plus signs along the antigen gene, while those sequence regions that have a negative effect are shown as minus signs. A pool of related antigen genes are non-stochastically generated using the methods described herein and screened to obtain those evolved nucleic acids that have gained positive sequence regions and lost negative regions. No pre-existing knowledge as to which regions are positive or negative for a particular trait is required.

15

**Figure 18: Screening strategy for antigen library screening.**

Shown here is a schematic representation of the screening strategy for antigen library screening.

20

**Figure 19: Strategy for pooling and deconvolution as used in antigen library screening.**

Shown here is a schematic representation of a strategy for pooling and deconvolution as used in antigen library screening.

25

**Figure 20: Preferred Embodiments of Site-Saturation Mutagenesis.**

**Figure 21. Schematic representation of a multimodule genetic vaccine vector.**

Shown here is a schematic representation of a multimodule genetic vaccine vector. A typical genetic vaccine vector will include one or more of the components indicated, each of which can be native or optimized using the directed evolution methods described herein. These directed evolution methods can include the introduction of point mutations by stochastic methods &/or by non-stochastic methods, including "gene site saturation mutagenesis" as described herein. These directed evolution methods can also include stochastic polynucleotide reassembly methods, for example by interrupted synthesis (as described in US5965408). These directed evolution methods can also include non-stochastic polynucleotide reassembly methods as described herein, including synthetic ligation polynucleotide reassembly as described herein. The components can be present on the same vaccine vector, or can be included in a genetic vaccine as separate molecules.

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**Figure 22A and Figure 22B. Generation of vectors with multiple T cell epitopes.**

Shown here are two different strategies for generating vectors that contain multiple T cell epitopes obtained, for example, by directed evolution. In Figure 60A, each individual non-stochastically generated epitope-encoding gene is linked to a single promoter, and multiple promoter-epitope gene constructs can be placed in a single vector. The scheme shown, described &/or referenced herein (including incorporated by reference) involves linking multiple epitope-encoding genes to a single promoter.

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**Figure 23. Generation of optimized genetic vaccines by directed evolution.**

Shown here is a diagram of the application of directed evolution to the generation of optimized genetic vaccines. Different forms of polynucleotides having known functional properties (e.g., regulatory, coding, and the like) are evolved and

screened to identify variants that exhibit improved properties for use as genetic vaccines.

- 5 **Figure 24. Recursive application of directed evolution and selection of evolved promoter sequences as an example of flow cytometry-based screening methods.** Shown here is a diagram of flow cytometry-based screening methods (FACS) for selection of optimized promoter sequences evolved using recursive applications of the directed evolution methods as described herein. A  
10 cytomegalovirus (CMV) promoter is used for illustrative purposes.

- Figure 25. An apparatus for microinjections of skin and muscle.** Shown here is an apparatus that is suitable for microinjection of genetic vaccines and other  
15 reagents into tissue such as skin and muscle. The apparatus is particularly useful for screening large numbers of agents in vivo, being based on a 96-well format. The tips of the apparatus are movable to allow adjustment so that the tips fit into a microtiter plate. After obtaining a reagent of interest is obtained from a plate, the tips are adjusted to a distance of about 2-3 mm apart, enabling transfer of 96  
20 different samples to an area of about 1.6 cm by 2.4 cm to about 2.4 cm by 3.6 cm. If desired, the volume of each sample transferred can be electronically controlled; typically the volumes transferred range from about 2  $\mu$ l to about 5  $\mu$ l. Each reagent can be mixed with a marker agent or dye to facilitate recognition of the injection site in the tissue. For example, gold particles of different sizes and  
25 shaped can be mixed with the reagent of interest, and microscopy and immunohistochemistry used to identify each injection site and to study the reaction induced by each reagent. When muscle tissue is injected, the injection site is first revealed by surgery.

**Figure 26. Polynucleotide reassembly.** Shown in Panel A is an example of directed evolution. n different strains of a virus are used in this illustration, but the technique is applicable to any single nucleic acid as well as to any nucleic acid for which different strains, species, or gene families have homologous nucleic acids that have one or more nucleotide changes compared to other homologous nucleic acids. The different variant nucleic acids are experimentally generated, preferably non-stochastically, as described herein, and screened or selected to identify those variants that exhibit the desired property. The directed evolution method(s) and screening can be repeated one or more times to obtain further improvement. Panel B shows that successive rounds of directed evolution can produce progressively enhanced properties, and that the combination of individual beneficial mutations can lead to an enhance improvement compared to the improvement achieved by an individual beneficial mutation.

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**Figure 27. Vector for promoter evolution.** Shown here is an example of a vector that is useful for screening to identify improved promoters from a library of promoter nucleic acids evolved using the directed evolution methods as described herein. Experimentally generated putative promoters are inserted into the vector upstream of a reporter gene for which expression is readily detected. For many applications, it is desirable that the product of the reporter gene be a cell surface protein so that cells which express high levels of the reporter gene can be sorted using flow cytometry-based cell sorting using the reporter gene product. Examples of suitable reporter genes include, for example, B7-2 and mAb179 epitopes. A polyadenylation region is typically placed downstream of the reporter gene (SV40 polyA is illustrated). The vector can also include a second reporter gene an internal control (GFP; green fluorescent protein); this gene is linked to a promoter (SR p). The vector also typically includes a selectable marker

25

(kanamycin/neomycin resistance is shown), and origins of replication that are functional in mammalian (SV40 ori) and/or bacterial (pUC ori) cells.

- 5 **Figure 28. Iterative evolution of inducible promoters using directed evolution and flow cytometry-based selection.** Shown here is a diagram of a scheme for iterative evolution of inducible promoters using the directed evolution methods as described herein and flow cytometry-based selection. A library of experimentally generated (i.e. produced by one or more directed evolution
- 10 methods as described herein) promoter nucleic acids present in appropriate vectors is transfected into the cells, and those cells which exhibit the least expression of marker antigen when grown under uninduced conditions are selected. The vectors (&/or cells containing them) are recovered, and the vectors are introduced into cells (if not contained therein already), and grown under inducing conditions.
- 15 Those cells that express the highest level of marker antigen are selected.

- Figure 29. Evolving a genetic vaccine vector for Oral, Intravenous, Intramuscular, Intradermal, Anal, Vaginal, or Topical Delivery.** Illustrated is
- 20 a strategy for screening of M13 libraries (e.g. generated experimentally using directed evolution as described herein) for desired targeting of various tissues. The particular example shown here is a schematic diagram of a method for evolving a genetic vaccine vector for improved oral delivery. This may comprise selecting for stability under the acidic conditions of the stomach, and resistance to other
- 25 degradatory factors of the digestive tract. The particular example illustrated relates to screening for improved oral delivery, but the same principle applies to libraries administered by other routes, including intravenously, intramuscularly, intradermally, anally, vaginally, or topically. After delivery to a test animal, the



M13 phage (or a product thereof) is recovered from the tissue of interest. The procedure can be repeated to obtain further optimization.

- 5    **Figure 30. An alignment of the nucleotide sequences of two human CMV strains and one monkey strain.** Shown here is an alignment of the nucleotide sequences of two human cytomegalovirus (CMV) strains and one monkey (Rhesus) strains. This alignment is serviceable for performing non-stochastic polynucleotide reassembly. Nucleotide sequences shared by 2 sequences are in blue lettering & nucleotide sequences shared by 3 sequences are in red lettering to  
10    illustrate preferred but non-limiting examples of reassembly points.

- 15    **Figure 31. An alignment of IL-4 nucleotide sequences from 3 species (human, primate, and canine).** Shown here is an alignment of the IL-4 nucleotide sequences of human, dog and primate strains. This alignment is serviceable for performing non-stochastic polynucleotide reassembly. Nucleotide sequences shared by 2 sequences are in blue lettering & nucleotide sequences shared by 3 sequences are in red lettering to illustrate preferred but non-limiting  
20    examples of reassembly points.

**Figure 32. Evolution of polypeptides by synthesizing (in vivo or in vitro) corresponding deduced polynucleotides and subjecting the deduced polynucleotides to directed evolution and expression screening subsequently expressed polypeptides.**

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**Figure 33. Non-stochastic Reassembly of oligo-directed CpG knock-outs.**

Shown here is a schematic representation of the use of the non-stochastic methods described herein to generate promoter sequences in which unnecessary CpG sequences are deleted, potentially useful CpG sequences are added, and non-replaceable CpG sequences are identified. Additionally, other sequences (aside from the CpG sequences) can be substituted into, added to, &/or deleted from working polynucleotides.

10 **Figure 34. An Example of a CTIS obtained from HbsAg polypeptide (PreS2 plus S regions).** Shown here is an example of a cytotoxic T-cell inducing sequence (CTIS) obtained from HBsAg polypeptide (PreS2 plus S regions).

20 **Figure 35. A CTIS Having Heterologous Epitopes Attached to the Cytoplasmic Portion.** Shown here is a CTIS having heterologous epitopes attached to the cytoplasmic portion.

25 **Figure 36. Method for preparing immunogenic agonist sequences (IAS).** Shown here is a method for preparing immunogenic agonist sequences (IAS). Wild-type (WT) and mutated forms of nucleic acids encoding a polypeptide of interest are assembled and subjected to non-stochastic reassembly to obtain a

nucleic acid encoding a poly-epitope region that contains potential agonist sequences.

- 5 **Figure 37. Improving Immunostimulatory Sequences (ISS) Using Directed Evolution.** Shown here is a scheme for improving immunostimulatory sequences by the directed evolution methods described herein. Oligonucleotide building blocks (e.g. synthetically generated), oligos with known ISS, CpG containing hexamers &/or oligos containing CpG containing hexamers, poly A, C, G, T,  
10 etc...can be assembled. The resultant molecule(s) can then be subjected to 1 or more directed evolution methods as described herein.

- Figure 38. Screening to identify IL-12 genes that encode recombinant IL-12**  
15 **having an increased ability to induce T Cell proliferation.** Shown here is a diagram of a procedure by which experimentally generated molecules, e.g. non-stochastically generated libraries of human IL-12 genes can be screened to identify evolved IL-12 genes that encode evolved forms of IL-12 having increased ability to induce T cell proliferation.

20

- Figure 39. Model of induction of T cell activation or anergy by genetic vaccine vectors encoding different CD80 and/or CD86 variants.** Shown here is a model of how T cell activation or anergy can be induced by genetic vaccine  
25 vectors that encode different B7-1 (CD80) and/or B7-2 (CD86) variants.

**Figure 40. Screening of CD80/CD86 variants that have improved capacity to induce T cell activation or anergy.** Shown here is a method for using directed evolution as described herein to obtain CD80/CD86 variants that have improved capacity to induce T cell activation or anergy.

5

**Figure 41. An alignment of two CMV-derived nucleotide sequences from human and primate species.** Shown here is an alignment of two CMV-derived nucleotide sequences of human and primate strains. This alignment is serviceable for performing non-stochastic polynucleotide reassembly. Nucleotide sequences shared by 2 sequences are in red lettering to illustrate preferred but non-limiting examples of reassembly points.

15

**Figure 42: An alignment of the IFN-gamma nucleotide sequences from human, cat, rodent species.** Shown here is an alignment of the IFN-gama nucleotide sequences from human, cat, and rodent species. This alignment is serviceable for performing non-stochastic polynucleotide reassembly. Nucleotide sequences shared by 2 sequences are in blue lettering & nucleotide sequences shared by 3 sequences are in red lettering to illustrate preferred but non-limiting examples of reassembly points.

20

## 2. DETAILED DESCRIPTION OF THE INVENTION

### 2.1. DEFINITIONS OF TERMS

5           In order to facilitate understanding of the examples provided herein, certain frequently occurring methods and/or terms will be described.

          The term "**agent**" is used herein to denote a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds (e.g.,  
10   a VLSIPS peptide array, polynucleotide array, and/or combinatorial small molecule array), biological macromolecule, a bacteriophage peptide display library, a bacteriophage antibody (e.g., scFv) display library, a polysome peptide display library, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particular mammalian) cells or tissues. Agents are  
15   evaluated for potential activity as anti-neoplastics, anti-inflammatories or apoptosis modulators by inclusion in screening assays described hereinbelow. Agents are evaluated for potential activity as specific protein interaction inhibitors (i.e., an agent which selectively inhibits a binding interaction between two predetermined polypeptides but which does not substantially interfere with cell  
20   viability) by inclusion in screening assays described hereinbelow.

          An "**ambiguous base requirement**" in a restriction site refers to a nucleotide base requirement that is not specified to the fullest extent, i.e. that is not a specific base (such as, in a non-limiting exemplification, a specific base  
25   selected from A, C, G, and T), but rather may be any one of at least two or more bases. Commonly accepted abbreviations that are used in the art as well as herein to represent ambiguity in bases include the following: **R** = G or A; **Y** = C or T; **M** = A or C; **K** = G or T; **S** = G or C; **W** = A or T; **H** = A or C or T; **B** = G or T or C; **V** = G or C or A; **D** = G or A or T; **N** = A or C or G or T.

“Alignment” with respect to molecular sequences is a way to determine similarity between 2 or more sequences. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., *infra*).

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative

score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of  
5 either sequence is reached.

The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100,  
10 M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915).

15 In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993) Proc. Nat'l. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a  
20 match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0. 1, more preferably less than about 0.0 1, and most preferably less than about 0.001.

25 Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to", refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent

conditions when that sequence is present in a complex mixture (e.g., total cellular DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

The term "**amino acid**" as used herein refers to any organic compound that contains an amino group ( $-NH_2$ ) and a carboxyl group ( $-COOH$ ); preferably either as free groups or alternatively after condensation as part of peptide bonds. The "**twenty naturally encoded polypeptide-forming alpha-amino acids**" are understood in the art and refer to: alanine (ala or A), arginine (arg or R), asparagine (asn or N), aspartic acid (asp or D), cysteine (cys or C), glutamic acid (glu or E), glutamine (gln or Q), glycine (gly or G), histidine (his or H), isoleucine (ile or I), leucine (leu or L), lysine (lys or K), methionine (met or M), phenylalanine (phe or F), proline (pro or P), serine (ser or S), threonine (thr or T), tryptophan (trp or W), tyrosine (tyr or Y), and valine (val or V).

The term "**amplification**" means that the number of copies of a polynucleotide is increased.

The term "**antibody**", as used herein, refers to intact immunoglobulin molecules, as well as fragments of immunoglobulin molecules, such as Fab, Fab', (Fab')<sub>2</sub>, Fv, and SCA fragments, that are capable of binding to an epitope of an antigen. These antibody fragments, which retain some ability to selectively bind to an antigen (e.g., a polypeptide antigen) of the antibody from which they are derived, can be made using well known methods in the art (see, e.g., **Harlow and Lane, supra**), and are described further, as follows.



- 5
- (1) An Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.
- 10
- (2) An Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.
- 15
- (3) An (Fab')<sub>2</sub> fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A (Fab')<sub>2</sub> fragment is a dimer of two Fab' fragments, held together by two disulfide bonds.
- 20
- (4) An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.
- 25
- (5) An single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

The term "Applied Molecular Evolution" ("AME") means the application of an evolutionary design algorithm to a specific, useful goal. While many different library formats for AME have been reported for polynucleotides, peptides and proteins (phage, lacI and polysomes), none of these formats have

provided for recombination by random cross-overs to deliberately create a combinatorial library.

A molecule that has a "**chimeric property**" is a molecule that is: 1) in part homologous and in part heterologous to a first reference molecule; while 2) at the same time being in part homologous and in part heterologous to a second reference molecule; without 3) precluding the possibility of being at the same time in part homologous and in part heterologous to still one or more additional reference molecules. In a non-limiting embodiment, a chimeric molecule may be prepared by assembling a reassortment of partial molecular sequences. In a non-limiting aspect, a chimeric polynucleotide molecule may be prepared by synthesizing the chimeric polynucleotide using plurality of molecular templates, such that the resultant chimeric polynucleotide has properties of a plurality of templates.

15

The term "**cognate**" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example, but not limitation, in the human genome the human CD4 gene is the cognate gene to the mouse 3d4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition.

A "**comparison window**," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of

sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith (**Smith and Waterman**, *Adv Appl Math*, 1981; **Smith and Waterman**, *J Teor Biol*, 1981; **Smith and Waterman**, *J Mol Biol*, 1981; **Smith et al**, *J Mol Evol*, 1981), by the homology alignment algorithm of Needleman (**Needleman and Wuncsch**, 1970), by the search of similarity method of Pearson (Pearson and Lipman, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

As used herein, the term "**complementarity-determining region**" and "**CDR**" refer to the art-recognized term as exemplified by the Kabat and Chothia CDR definitions also generally known as supervariable regions or hypervariable loops (**Chothia and Lesk**, 1987; **Chothia et al**, 1989; **Kabat et al**, 1987; and **Tramontano et al**, 1990). Variable region domains typically comprise the amino-terminal approximately 105-115 amino acids of a naturally-occurring immunoglobulin chain (e.g., amino acids 1-110), although variable domains somewhat shorter or longer are also suitable for forming single-chain antibodies.

"**Conservative amino acid substitutions**" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-

containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are : valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

5           **"Conservatively modified variations"** of a particular polynucleotide sequence refers to those polynucleotides that encode identical or essentially identical amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic  
10 acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CCG, AGA, and AGG all encode the amino acid arginine.

Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without  
15 altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every polynucleotide sequence described herein which encodes a polypeptide also describes every possible silent variation, except where otherwise noted.

20           One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

25

Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are **"conservatively modified variations"** where the

alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another:

- 5           Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I);
- Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- Sulfur-containing: Methionine (M), Cysteine (C);
- Basic: Arginine (R), Lysine (K), Histidine (H);
- 10          Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q).

See also, Creighton (1984) Proteins, W.H. Freeman and Company, for additional groupings of amino acids. In addition, individual substitutions,

15   deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "**conservatively modified variations**".

The term "**corresponds to**" is used herein to mean that a polynucleotide

20   sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide

25   sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference "TATAC" and is complementary to a reference sequence "GTATA."

The term "**cytokine**" includes, for example, interleukins, interferons, chemokines, hematopoietic growth factors, tumor necrosis factors and

transforming growth factors. In general these are small molecular weight proteins that regulate maturation, activation, proliferation and differentiation of the cells of the immune system.

5           The term "**degrading effective**" amount refers to the amount of enzyme which is required to process at least 50% of the substrate, as compared to substrate not contacted with the enzyme. Preferably, at least 80% of the substrate is degraded.

10           As used herein, the term "**defined sequence framework**" refers to a set of defined sequences that are selected on a non-random basis, generally on the basis of experimental data or structural data; for example, a defined sequence framework may comprise a set of amino acid sequences that are predicted to form a  $\beta$ -sheet structure or may comprise a leucine zipper heptad repeat motif, a zinc-  
15   finger domain, among other variations. A "defined sequence kernal" is a set of sequences which encompass a limited scope of variability. Whereas (1) a completely random 10-mer sequence of the 20 conventional amino acids can be any of  $(20)^{10}$  sequences, and (2) a pseudorandom 10-mer sequence of the 20 conventional amino acids can be any of  $(20)^{10}$  sequences but will exhibit a bias  
20   for certain residues at certain positions and/or overall, (3) a defined sequence kernal is a subset of sequences if each residue position was allowed to be any of the allowable 20 conventional amino acids (and/or allowable unconventional amino/imino acids). A defined sequence kernal generally comprises variant and invariant residue positions and/or comprises variant residue positions which can  
25   comprise a residue selected from a defined subset of amino acid residues), and the like, either segmentally or over the entire length of the individual selected library member sequence. Defined sequence kernels can refer to either amino acid sequences or polynucleotide sequences. Of illustration and not limitation, the

sequences (NNK)<sub>10</sub> and (NNM)<sub>10</sub>, wherein N represents A, T, G, or C; K represents G or T; and M represents A or C, are defined sequence kernels.

"**Digestion**" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a gel to isolate the desired fragment.

"**Directional ligation**" refers to a ligation in which a 5' end and a 3' end of a polynucleotide are different enough to specify a preferred ligation orientation. For example, an otherwise untreated and undigested PCR product that has two blunt ends will typically not have a preferred ligation orientation when ligated into a cloning vector digested to produce blunt ends in its multiple cloning site; thus, directional ligation will typically not be displayed under these circumstances. In contrast, directional ligation will typically displayed when a digested PCR product having a 5' *EcoR* I-treated end and a 3' *BamH* I-is ligated into a cloning vector that has a multiple cloning site digested with *EcoR* I and *BamH* I.

The term "**DNA shuffling**" is used herein to indicate recombination between substantially homologous but non-identical sequences, in some embodiments DNA shuffling may involve crossover via non-homologous recombination, such as via cer/lox and/or flp/ftt systems and the like.

5

As used in this invention, the term "**epitope**" refers to an antigenic determinant on an antigen, such as a phytase polypeptide, to which the paratope of an antibody, such as an phytase-specific antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. As used herein "**epitope**" refers to that portion of an antigen or other macromolecule capable of forming a binding interaction that interacts with the variable region binding body of an antibody. Typically, such binding interaction is manifested as an intermolecular contact with one or more amino acid residues of a CDR.

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An "**exogenous DNA segment**", "**heterologous sequence**" or a "**heterologous nucleic acid**", as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell, but has been modified. Modification of a heterologous sequence in the applications described herein typically occurs through the use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly. Thus, the terms refer to a DNA segment which is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found.

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"Exogenous" DNA segments are expressed to yield exogenous polypeptides.

The term "gene" is used broadly to refer to any segment of DNA associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

10

An "experimentally generated (in vitro &/or in vivo) polynucleotide" (which term includes a "recombinant polynucleotide") or an "experimentally (in vitro &/or in vivo) generated polypeptide" (which term includes a "experimentally generated polypeptide") is a non-naturally occurring polynucleotide or polypeptide that includes nucleic acid or amino acid sequences, respectively, from more than one source nucleic acid or polypeptide, which source nucleic acid or polypeptide can be a naturally occurring nucleic acid or polypeptide, or can itself have been subjected to mutagenesis or other type of modification. The source polynucleotides or polypeptides from which the different nucleic acid or amino acid sequences are derived are sometimes homologous (i.e., have, or encode a polypeptide that encodes, the same or a similar structure and/or function), and are often from different isolates, serotypes, strains, species, of organism or from different disease states, for example.

20

The terms "fragment", "derivative" and "analog" when referring to a reference polypeptide comprise a polypeptide which retains at least one biological function or activity that is at least essentially same as that of the reference polypeptide. Furthermore, the terms "fragment", "derivative" or "analog" are exemplified by a "pro-form" molecule, such as a low activity proprotein that can

25

be modified by cleavage to produce a mature enzyme with significantly higher activity.

5 A method is provided herein for producing from a template polypeptide a set of progeny polypeptides in which a **"full range of single amino acid substitutions"** is represented at each amino acid position. As used herein, **"full range of single amino acid substitutions"** is in reference to the naturally encoded 20 naturally encoded polypeptide-forming alpha-amino acids, as described herein.

10

The term **"gene"** means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

15

**"Genetic instability"**, as used herein, refers to the natural tendency of highly repetitive sequences to be lost through a process of reductive events generally involving sequence simplification through the loss of repeated sequences. Deletions tend to involve the loss of one copy of a repeat and  
20 everything between the repeats.

The term **"heterologous"** means that one single-stranded nucleic acid sequence is unable to hybridize to another single-stranded nucleic acid sequence or its complement. Thus areas of heterology means that areas of polynucleotides  
25 or polynucleotides have areas or regions within their sequence which are unable to hybridize to another nucleic acid or polynucleotide. Such regions or areas are for example areas of mutations.

The term "**homologous**" or "**homeologous**" means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentrations as discussed later. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, also called CDR's. The extent of the framework region and CDR's have been precisely defined; see "Sequences of Proteins of Immunological Interest" (Kabat et al, 1987). The sequences of the framework regions of different light or heavy chains are relatively conserved within a specie. As used herein, a "**human framework region**" is a framework region that is substantially identical (about 85 or more, usually 90-95 or more) to the framework region of a naturally occurring human immunoglobulin. the framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDR's. The CDR's are primarily responsible for binding to an epitope of an antigen.

The benefits of this invention extend to "**commercial applications**" (or commercial processes), which term is used to include applications in commercial industry proper (or simply industry) as well as non-commercial commercial applications (e.g. biomedical research at a non-profit institution). Relevant applications include those in areas of diagnosis, medicine, agriculture, manufacturing, and academia.

The term "**identical**" or "**identity**" means that two nucleic acid sequences have the same sequence or a complementary sequence. Thus, "areas of identity" means that regions or areas of a polynucleotide or the overall polynucleotide are identical or complementary to areas of another polynucleotide or the  
5 polynucleotide.

The terms "**identical**" or percent "**identity**," in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid  
10 residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

For sequence comparison, typically one sequence acts as a reference  
15 sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the  
20 reference sequence, based on the designated program parameters.

A further indication that two nucleic acid sequences or polypeptides are substantially "**identical**" is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with, or specifically binds to, the polypeptide  
25 encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions.

The term "**isolated**" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from  
5 some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

10 The term "**isolated**", when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such  
15 as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames which flank the gene and encode a protein other than the gene of interest.

20

By "**isolated nucleic acid**" is meant a nucleic acid, e.g., a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus  
25 describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, e.g., a DNA fragment produced by PCR amplification or restriction

enzyme digestion, or an RNA molecule produced by *in vitro* transcription. The term also describes a recombinant nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

5

As used herein "**ligand**" refers to a molecule, such as a random peptide or variable segment sequence, that is recognized by a particular receptor. As one of skill in the art will recognize, a molecule (or macromolecular complex) can be both a receptor and a ligand. In general, the binding partner having a smaller molecular weight is referred to as the ligand and the binding partner having a greater molecular weight is referred to as a receptor.

"**Ligation**" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Sambrook et al, 1982, p. 146; Sambrook, 1989). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

As used herein, "**linker**" or "**spacer**" refers to a molecule or group of molecules that connects two molecules, such as a DNA binding protein and a random peptide, and serves to place the two molecules in a preferred configuration, e.g., so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein.

As used herein, a "**molecular property to be evolved**" includes reference to molecules comprised of a polynucleotide sequence, molecules comprised of a polypeptide sequence, and molecules comprised in part of a polynucleotide sequence and in part of a polypeptide sequence. Particularly relevant - but by no means limiting - examples of molecular properties to be evolved include

enzymatic activities at specified conditions, such as related to temperature; salinity; pressure; pH; and concentration of glycerol, DMSO, detergent, &/or any other molecular species with which contact is made in a reaction environment.

- Additional particularly relevant - but by no means limiting - examples of
- 5 molecular properties to be evolved include stabilities - e.g. the amount of a residual molecular property that is present after a specified exposure time to a specified environment, such as may be encountered during storage.

- A "**multivalent antigenic polypeptide**" or a "**recombinant multivalent**
- 10 **antigenic polypeptide**" is a non-naturally occurring polypeptide that includes amino acid sequences from more than one source polypeptide, which source polypeptide is typically a naturally occurring polypeptide. At least some of the regions of different amino acid sequences constitute epitopes that are recognized by antibodies found in a mammal that has been injected with the source
- 15 polypeptide. The source polypeptides from which the different epitopes are derived are usually homologous (i.e., have the same or a similar structure and/or function), and are often from different isolates, serotypes, strains, species, of organism or from different disease states, for example.

- 20 The term "**mutations**" includes changes in the sequence of a wild-type or parental nucleic acid sequence or changes in the sequence of a peptide. Such mutations may be point mutations such as transitions or transversions. The mutations may be deletions, insertions or duplications. A mutation can also be a "**chimerization**", which is exemplified in a progeny molecule that is generated to
- 25 contain part or all of a sequence of one parental molecule as well as part or all of a sequence of at least one other parental molecule. This invention provides for both chimeric polynucleotides and chimeric polypeptides.

As used herein, the degenerate "N,N,G/T" nucleotide sequence represents 32 possible triplets, where "N" can be A, C, G or T.

The term "**naturally-occurring**" as used herein as applied to the object  
5 refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses bacteria, protozoa, insects, plants or mammalian tissue) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring. Generally, the term naturally  
10 occurring refers to an object as present in a non-pathological (un-diseased) individual, such as would be typical for the species.

The term "**nucleic acid**" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless  
15 specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof  
20 (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al. (1991) Nucleic Acid Res. 19: 5081; Ohtsuka et al. (1985) J  
25 Biol. Chem. 260: 2605-2608; Cassol et al. (1992) Rossolini et al. (1994) Mol. Cell. Probes 8: 91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.



"Nucleic acid derived from a gene" refers to a nucleic acid for whose synthesis the gene, or a subsequence thereof, has ultimately served as a template. Thus, an mRNA, a cDNA reverse transcribed from an mRNA, an RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA  
5 transcribed from the amplified DNA, etc., are all derived from the gene and detection of such derived products is indicative of the presence and/or abundance of the original gene and/or gene transcript in a sample.

As used herein, a "nucleic acid molecule" is comprised of at least one  
10 base or one base pair, depending on whether it is single-stranded or double-stranded, respectively. Furthermore, a nucleic acid molecule may belong exclusively or chimerically to any group of nucleotide-containing molecules, as exemplified by, but not limited to, the following groups of nucleic acid molecules: RNA, DNA, genomic nucleic acids, non-genomic nucleic acids, naturally  
15 occurring and not naturally occurring nucleic acids, and synthetic nucleic acids. This includes, by way of non-limiting example, nucleic acids associated with any organelle, such as the mitochondria, ribosomal RNA, and nucleic acid molecules comprised chimerically of one or more components that are not naturally occurring along with naturally occurring components.

20

Additionally, a "nucleic acid molecule" may contain in part one or more non-nucleotide-based components as exemplified by, but not limited to, amino acids and sugars. Thus, by way of example, but not limitation, a ribozyme that is in part nucleotide-based and in part protein-based is considered a "nucleic acid  
25 molecule".

In addition, by way of example, but not limitation, a nucleic acid molecule that is labeled with a detectable moiety, such as a radioactive or alternatively a non-radioactive label, is likewise considered a "nucleic acid molecule".

The terms **"nucleic acid sequence coding for"** or a **"DNA coding sequence of"** or a **"nucleotide sequence encoding"** a particular enzyme – as well as other synonymous terms – refer to a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences. A **"promotor sequence"** is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter is part of the DNA sequence. This sequence region has a start codon at its 3' terminus. The promoter sequence does include the minimum number of bases where elements necessary to initiate transcription at levels detectable above background. However, after the RNA polymerase binds the sequence and transcription is initiated at the start codon (3' terminus with a promoter), transcription proceeds downstream in the 3' direction. Within the promotor sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1) as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The terms **"nucleic acid encoding an enzyme (protein)"** or **"DNA encoding an enzyme (protein)"** or **"polynucleotide encoding an enzyme (protein)"** and other synonymous terms encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

In one preferred embodiment, a **"specific nucleic acid molecule species"** is defined by its chemical structure, as exemplified by, but not limited to, its primary sequence. In another preferred embodiment, a specific **"nucleic acid molecule species"** is defined by a function of the nucleic acid species or by a function of a product derived from the nucleic acid species. Thus, by way of non-limiting example, a **"specific nucleic acid molecule species"** may be defined by

one or more activities or properties attributable to it, including activities or properties attributable its expressed product.

The instant definition of **"assembling a working nucleic acid sample into a nucleic acid library"** includes the process of incorporating a nucleic acid sample into a vector-based collection, such as by ligation into a vector and transformation of a host. A description of relevant vectors, hosts, and other reagents as well as specific non-limiting examples thereof are provided hereinafter. The instant definition of **"assembling a working nucleic acid sample into a nucleic acid library"** also includes the process of incorporating a nucleic acid sample into a non-vector-based collection, such as by ligation to adaptors. Preferably the adaptors can anneal to PCR primers to facilitate amplification by PCR.

Accordingly, in a non-limiting embodiment, a **"nucleic acid library"** is comprised of a vector-based collection of one or more nucleic acid molecules. In another preferred embodiment a **"nucleic acid library"** is comprised of a non-vector-based collection of nucleic acid molecules. In yet another preferred embodiment a **"nucleic acid library"** is comprised of a combined collection of nucleic acid molecules that is in part vector-based and in part non-vector-based. Preferably, the collection of molecules comprising a library is searchable and separable according to individual nucleic acid molecule species.

The present invention provides a **"nucleic acid construct"** or alternatively a **"nucleotide construct"** or alternatively a **"DNA construct"**. The term "construct" is used herein to describe a molecule, such as a polynucleotide (*e.g.*, a phytase polynucleotide) may optionally be chemically bonded to one or more additional molecular moieties, such as a vector, or parts of a vector. In a specific - but by no means limiting - aspect, a nucleotide construct is exemplified by a DNA

expression DNA expression constructs suitable for the transformation of a host cell.

An "oligonucleotide" (or synonymously an "oligo") refers to either a  
5 single stranded polydeoxynucleotide or two complementary polydeoxynucleotide  
strands which may be chemically synthesized. Such synthetic oligonucleotides  
may or may not have a 5' phosphate. Those that do not will not ligate to another  
oligonucleotide without adding a phosphate with an ATP in the presence of a  
kinase. A synthetic oligonucleotide will ligate to a fragment that has not been  
10 dephosphorylated. To achieve polymerase-based amplification (such as with  
PCR), a **"32-fold degenerate oligonucleotide that is comprised of, in series, at  
least a first homologous sequence, a degenerate N,N,G/T sequence, and a  
second homologous sequence"** is mentioned. As used in this context,  
"homologous" is in reference to homology between the oligo and the parental  
15 polynucleotide that is subjected to the polymerase-based amplification.

A nucleic acid is **"operably linked"** when it is placed into a functional  
relationship with another nucleic acid sequence. For instance, a promoter or  
enhancer is operably linked to a coding sequence if it increases the transcription  
20 of the coding sequence.

As used herein, the term **"operably linked"** refers to a linkage of  
polynucleotide elements in a functional relationship. A nucleic acid is **"operably  
linked"** when it is placed into a functional relationship with another nucleic acid  
25 sequence. For instance, a promoter or enhancer is operably linked to a coding  
sequence if it affects the transcription of the coding sequence. Operably linked  
means that the DNA sequences being linked are typically contiguous and, where  
necessary to join two protein coding regions, contiguous and in reading frame.  
However, since enhancers generally function when separated from the promoter

by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

A coding sequence is "**operably linked to**" another coding sequence when  
5 RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein.

10

As used herein the term "**parental polynucleotide set**" is a set comprised of one or more distinct polynucleotide species. Usually this term is used in reference to a progeny polynucleotide set which is preferably obtained by mutagenization of the parental set, in which case the terms "**parental**", "**starting**"  
15 and "**template**" are used interchangeably.

As used herein the term "**physiological conditions**" refers to temperature, pH, ionic strength, viscosity, and like biochemical parameters which are compatible with a viable organism, and/or which typically exist intracellularly in  
20 a viable cultured yeast cell or mammalian cell. For example, the intracellular conditions in a yeast cell grown under typical laboratory culture conditions are physiological conditions. Suitable *in vitro* reaction conditions for *in vitro* transcription cocktails are generally physiological conditions. In general, *in vitro* physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45°C  
25 and 0.001-10 mM divalent cation (e.g.,  $Mg^{++}$ ,  $Ca^{++}$ ); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (e.g., BSA). A non-ionic detergent (Tween, NP-40, Triton X-100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (v/v). Particular aqueous conditions may be selected by the practitioner

according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents and/or membrane fractions and/or anti-foam agents and/or  
5 scintillants.

Standard convention (5' to 3') is used herein to describe the sequence of double stranded polynucleotides.

10 The term "**population**" as used herein means a collection of components such as polynucleotides, portions or polynucleotides or proteins. A "mixed population: means a collection of components which belong to the same family of nucleic acids or proteins (i.e., are related) but which differ in their sequence (i.e., are not identical) and hence in their biological activity.

15 A molecule having a "**pro-form**" refers to a molecule that undergoes any combination of one or more covalent and noncovalent chemical modifications (e.g. glycosylation, proteolytic cleavage, dimerization or oligomerization, temperature-induced or pH-induced conformational change, association with a co-  
20 factor, etc.) en route to attain a more mature molecular form having a property difference (e.g. an increase in activity) in comparison with the reference pro-form molecule. When two or more chemical modification (e.g. two proteolytic cleavages, or a proteolytic cleavage and a deglycosylation) can be distinguished en route to the production of a mature molecule, the reference precursor molecule  
25 may be termed a "**pre-pro-form**" molecule.

As used herein, the term "**pseudorandom**" refers to a set of sequences that have limited variability, such that, for example, the degree of residue variability at

another position, but any pseudorandom position is allowed some degree of residue variation, however circumscribed.

5       The term "**purified**" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

10       "**Quasi-repeated units**", as used herein, refers to the repeats to be reassorted and are by definition not identical. Indeed the method is proposed not only for practically identical encoding units produced by mutagenesis of the identical starting sequence, but also the reassortment of similar or related sequences which may diverge significantly in some regions. Nevertheless, if the sequences contain sufficient homologies to be reassorted by this approach, they  
15       can be referred to as "quasi-repeated" units.

      As used herein "**random peptide library**" refers to a set of polynucleotide sequences that encodes a set of random peptides, and to the set of random peptides encoded by those polynucleotide sequences, as well as the fusion  
20       proteins contain those random peptides.

      As used herein, "**random peptide sequence**" refers to an amino acid sequence composed of two or more amino acid monomers and constructed by a stochastic or random process. A random peptide can include framework or  
25       scaffolding motifs, which may comprise invariant sequences.

      As used herein, "**receptor**" refers to a molecule that has an affinity for a given ligand. Receptors can be naturally occurring or synthetic molecules. Receptors can be employed in an unaltered state or as aggregates with other

species. Receptors can be attached, covalently or non-covalently, to a binding member, either directly or via a specific binding substance. Examples of receptors include, but are not limited to, antibodies, including monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on  
5 viruses, cells, or other materials), cell membrane receptors, complex carbohydrates and glycoproteins, enzymes, and hormone receptors.

The term "**recombinant**" when used with reference to a cell indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein  
10 encoded by a heterologous nucleic acid. Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell  
15 that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

"**Recombinant enzymes**" refer to enzymes produced by recombinant  
20 DNA techniques, i.e., produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "**Synthetic**" enzymes are those prepared by chemical synthesis.

A "**recombinant expression cassette**" or simply an "**expression cassette**"  
25 is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (e.g., a nucleic acid



encoding a desired polypeptide), and a promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein from the host cell.

- 5 Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

The term "**related polynucleotides**" means that regions or areas of the polynucleotides are identical and regions or areas of the polynucleotides are  
10 heterologous.

"**Reductive reassortment**", as used herein, refers to the increase in molecular diversity that is accrued through deletion (and/or insertion) events that are mediated by repeated sequences.

15

The following terms are used to describe the sequence relationships between two or more polynucleotides: "**reference sequence**," "**comparison window**," "**sequence identity**," "**percentage of sequence identity**," and "**substantial identity**."

20

A "**reference sequence**" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence.

- 25 Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides and (2) may further comprise a sequence that is divergent

between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

5

**"Repetitive Index (RI)"**, as used herein, is the average number of copies of the quasi-repeated units contained in the cloning vector.

The term **"restriction site"** refers to a recognition sequence that is  
10 necessary for the manifestation of the action of a restriction enzyme, and includes a site of catalytic cleavage. It is appreciated that a site of cleavage may or may not be contained within a portion of a restriction site that comprises a low ambiguity sequence (i.e. a sequence containing the principal determinant of the frequency of occurrence of the restriction site). Thus, in many cases, relevant  
15 restriction sites contain only a low ambiguity sequence with an internal cleavage site (e.g. G/AATTC in the EcoR I site) or an immediately adjacent cleavage site (e.g. /CCWGG in the EcoR II site). In other cases, relevant restriction enzymes [e.g. the Eco57 I site or CTGAAG(16/14)] contain a low ambiguity sequence (e.g. the CTGAAG sequence in the Eco57 I site) with an external cleavage site (e.g. in  
20 the N<sub>16</sub> portion of the Eco57 I site). When an enzyme (e.g. a restriction enzyme) is said to **"cleave"** a polynucleotide, it is understood to mean that the restriction enzyme catalyzes or facilitates a cleavage of a polynucleotide.

The term **"screening"** describes, in general, a process that identifies  
25 optimal antigens. Several properties of the antigen can be used in selection and screening including antigen expression, folding, stability, immunogenicity and presence of epitopes from several related antigens. Selection is a form of screening in which identification and physical separation are achieved simultaneously by expression of a selection marker, which, in some genetic

circumstances, allows cells expressing the marker to survive while other cells die (or vice versa). Screening markers include, for example, luciferase, beta-galactosidase and green fluorescent protein. Selection markers include drug and toxin resistance genes, and the like. Because of limitations in studying primary  
5 immune responses *in vitro*, *in vivo* studies are particularly useful screening methods. In these studies, the antigens are first introduced to test animals, and the immune responses are subsequently studied by analyzing protective immune responses or by studying the quality or strength of the induced immune response using lymphoid cells derived from the immunized animal. Although spontaneous  
10 selection can and does occur in the course of natural evolution, in the present methods selection is performed by man.

In a non-limiting aspect, a "selectable polynucleotide" is comprised of a 5' terminal region (or end region), an intermediate region (i.e. an internal or  
15 central region), and a 3' terminal region (or end region). As used in this aspect, a 5' terminal region is a region that is located towards a 5' polynucleotide terminus (or a 5' polynucleotide end); thus it is either partially or entirely in a 5' half of a polynucleotide. Likewise, a 3' terminal region is a region that is located towards a 3' polynucleotide terminus (or a 3' polynucleotide end); thus it is either partially  
20 or entirely in a 3' half of a polynucleotide. As used in this non-limiting exemplification, there may be sequence overlap between any two regions or even among all three regions.

The term "sequence identity" means that two polynucleotide sequences  
25 are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched

positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. This "substantial identity", as used herein, denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence having at least 80 percent sequence identity, preferably at least 85 percent identity, often 90 to 95 percent sequence identity, and most commonly at least 99 percent sequence identity as compared to a reference sequence of a comparison window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme. Similarity may be determined by procedures which are well-known in the art, for example, a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information).

20

As used herein, the term "single-chain antibody" refers to a polypeptide comprising a  $V_H$  domain and a  $V_L$  domain in polypeptide linkage, generally linked via a spacer peptide (e.g., [Gly-Gly-Gly-Gly-Ser]<sub>x</sub>), and which may comprise additional amino acid sequences at the amino- and/or carboxy- termini. For example, a single-chain antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a scFv is a single-chain antibody. Single-chain antibodies are generally proteins consisting of one or more polypeptide segments of at least 10 contiguous amino substantially encoded by genes of the immunoglobulin superfamily (e.g., see Williams and Barclay, 1989,

pp. 361-368, which is incorporated herein by reference), most frequently encoded by a rodent, non-human primate, avian, porcine bovine, ovine, goat, or human heavy chain or light chain gene sequence. A functional single-chain antibody generally contains a sufficient portion of an immunoglobulin superfamily gene product so as to retain the property of binding to a specific target molecule, typically a receptor or antigen (epitope).

The phrase "specifically (or selectively) binds to an antibody" or "specifically (or selectively) immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein, or an epitope from the protein, in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. The antibodies raised against a multivalent antigenic polypeptide will generally bind to the proteins from which one or more of the epitopes were obtained. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays, Western blots, or immunohistochemistry are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York ("Harlow and Lane"), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

The members of a pair of molecules (*e.g.*, an antibody-antigen pair or a nucleic acid pair) are said to "**specifically bind**" to each other if they bind to each other with greater affinity than to other, non-specific molecules. For example, an antibody raised against an antigen to which it binds more efficiently than to a non-specific protein can be described as specifically binding to the antigen.  
(Similarly, a nucleic acid probe can be described as specifically binding to a nucleic acid target if it forms a specific duplex with the target by base pairing interactions (see above).)

10 A "**specific binding affinity**" between two molecules, for example, a ligand and a receptor, means a preferential binding of one molecule for another in a mixture of molecules. The binding of the molecules can be considered specific if the binding affinity is about  $1 \times 10^4 \text{ M}^{-1}$  to about  $1 \times 10^6 \text{ M}^{-1}$  or greater.

15 "**Specific hybridization**" is defined herein as the formation of hybrids between a first polynucleotide and a second polynucleotide (*e.g.*, a polynucleotide having a distinct but substantially identical sequence to the first polynucleotide), wherein substantially unrelated polynucleotide sequences do not form hybrids in the mixture.

20 The term "**specific polynucleotide**" means a polynucleotide having certain end points and having a certain nucleic acid sequence. Two polynucleotides wherein one polynucleotide has the identical sequence as a portion of the second polynucleotide but different ends comprises two different specific  
25 polynucleotides.

The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the  $T_m$ , for a particular probe. An

example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight.

5

**"Stringent hybridization conditions"** means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. *See Sambrook et al, 1989, which is hereby incorporated by reference in its entirety.*

10

An example of highly **"stringent"** wash conditions is 0.15M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (see, Sambrook, *infra.*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0 M Na<sup>+</sup> ion, typically about 0.01 to 1.0 M Na<sup>+</sup> ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

25

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different  
5 under different environmental parameters. Longer sequences hybridize specifically at higher temperatures.

An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--  
10 Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent  
15 conditions" a probe will hybridize to its target subsequence, but to no other sequences.

Also included in the invention are polypeptides having sequences that are "substantially identical" to the sequence of a phytase polypeptide, such as one of  
20 SEQ ID 1. A "substantially identical" amino acid sequence is a sequence that differs from a reference sequence only by conservative amino acid substitutions, for example, substitutions of one amino acid for another of the same class (*e.g.*, substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such  
25 as substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine).

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at



least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the  
5 sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In some embodiments, the sequences are substantially identical over the entire length of the coding regions.

10 A "subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part of a longer sequence of nucleic acids or amino acids (e. g., polypeptide) respectively.

Additionally a "substantially identical" amino acid sequence is a  
15 sequence that differs from a reference sequence or by one or more non-conservative substitutions, deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site the molecule, and provided that the polypeptide essentially retains its behavioural properties. For example, one or more amino acids can be deleted from a phytase polypeptide, resulting in  
20 modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for phytase biological activity can be removed. Such modifications can result in the development of smaller active phytase polypeptides.

25

The present invention provides a "substantially pure enzyme". The term "substantially pure enzyme" is used herein to describe a molecule, such as a polypeptide (e.g., a phytase polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other

biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. The purity of the polypeptides can be determined using standard methods including, e.g., polyacrylamide gel electrophoresis (e.g., SDS-PAGE), column chromatography (e.g., high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

As used herein, "**substantially pure**" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual macromolecular species in the composition), and preferably substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

As used herein, the term "**variable segment**" refers to a portion of a nascent peptide which comprises a random, pseudorandom, or defined kernal sequence. A variable segment" refers to a portion of a nascent peptide which comprises a random pseudorandom, or defined kernal sequence. A variable segment can comprise both variant and invariant residue positions, and the degree of residue variation at a variant residue position may be limited: both options are selected at the discretion of the practitioner. Typically, variable segments are about 5 to 20 amino acid residues in length (e.g., 8 to 10), although variable

segments may be longer and may comprise antibody portions or receptor proteins, such as an antibody fragment, a nucleic acid binding protein, a receptor protein, and the like.

5           The term "**wild-type**" means that the polynucleotide does not comprise any mutations. A "wild type" protein means that the protein will be active at a level of activity found in nature and will comprise the amino acid sequence found in nature.

10           The term "**working**", as in "**working sample**", for example, is simply a sample with which one is working. Likewise, a "**working molecule**", for example is a molecule with which one is working.

15

## 2.2. GENERAL CONSIDERATIONS & FORMATS FOR RECOMBINATION

20           Component modules provides genetic vaccine with the acquisition of or improvement in a useful property or characteristic.

25           The present invention provides multicomponent genetic vaccines that include one or more component modules, each of which provides the genetic vaccine with the acquisition of or an improvement in a property or characteristic useful in genetic vaccination.

          The invention provides significant advantages over previously used genetic vaccines. Through use of a multicomponent vaccine, one can obtain an immune response that is particularly effective for a particular application. A

multicomponent genetic vaccine can, for example, contain a component that is optimized for optimal antigen expression, as well as a component that confers improved activation of cytotoxic T lymphocytes (CTLs) by enhancing the presentation of the antigen on dendritic cell MHC Class I molecules. Additional  
5 examples are described herein.

The invention provides a new approach to vaccine development, which is termed "antigen library immunization." No other technologies are available for generating libraries of related antigens or optimizing known protective antigens.  
10 The most powerful previously existing methods for identification of vaccine antigens, such as high throughput sequencing or expression library immunization, only explore the sequence space provided by the pathogen genome. These approaches are likely to be insufficient, because they generally only target single pathogen strains, and because natural evolution has directed pathogens to  
15 downregulate their own immunogenicity. In contrast, the immunization protocols of the invention, which use experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigen libraries, provide a means to identify novel antigen sequences. Those antigens that are most protective can be selected from these pools by *in vivo* challenge models. Antigen  
20 library immunization dramatically expands the diversity of available immunogen sequences, and therefore, these antigen chimera libraries can also provide means to defend against newly emerging pathogen variants of the future. The methods of the invention enable the identification of individual chimeric antigens that provide efficient protection against a variety of existing pathogens, providing improved  
25 vaccines for troops and civilian populations.

The methods of the invention provide an evolution-based approach, such as stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly in particular, that is an optimal approach to

improve the immunogenicity of many types of antigens. For example, the methods provide means of obtaining optimized cancer antigens useful for preventing and treating malignant diseases. Furthermore, an increasing number of self-antigens, causing autoimmune diseases, and allergens, causing atopy, allergy  
5 and asthma, have been characterized. The immunogenicity and manufacturing of these antigens can likewise be improved with the methods of this invention.

The antigen library immunization methods of the invention provide a means by which one can obtain a recombinant antigen that has improved ability to  
10 induce an immune response to a pathogenic agent. A "pathogenic agent" refers to an organism or virus that is capable of infecting a host cell. Pathogenic agents typically include and/or encode a molecule, usually a polypeptide, that is immunogenic in that an immune response is raised against the immunogenic polypeptide. Often, the immune response raised against an immunogenic  
15 polypeptide from one serotype of the pathogenic agent is not capable of recognizing, and thus protecting against, a different serotype of the pathogenic agent, or other related pathogenic agents. In other situations, the polypeptide produced by a pathogenic agent is not produced in sufficient amounts, or is not sufficiently immunogenic, for the infected host to raise an effective immune  
20 response against the pathogenic agent.

These problems are overcome by the methods of the invention, which typically involve reassembling (&/or subjecting to one or more directed evolution methods described herein) two or more forms of a nucleic acid that encode a  
25 polypeptide of the pathogenic agent, or antigen involved in another disease or condition. These reassembly methods, including stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, use as substrates forms of the nucleic acid that differ from each other in two or more nucleotides, so a library of recombinant nucleic acids results. The library is

then screened to identify at least one optimized recombinant nucleic acid that encodes an optimized recombinant antigen that has improved ability to induce an immune response to the pathogenic agent or other condition.

5           The resulting recombinant antigens often are chimeric in that they are recognized by antibodies (Abs) reacting against multiple pathogen strains, and generally can also elicit broad spectrum immune responses. Specific neutralizing antibodies are known to mediate protection against several pathogens of interest, although additional mechanisms, such as cytotoxic T lymphocytes, are likely to  
10 be involved. The concept of chimeric, multivalent antigens inducing broadly reacting antibody responses is further illustrated herein.

          In preferred embodiments, the different forms of the nucleic acids that encode antigenic polypeptides are obtained from members of a family of related  
15 pathogenic agents.

          This scheme of performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly using nucleic acids from different organisms is shown schematically herein. Therefore, these  
20 stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods provide an effective approach to generate multivalent, crossprotective antigens. The methods are useful for obtaining individual chimeras that effectively protect against most or all pathogen variants.

25

          Moreover, immunizations using entire libraries or pools of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigen chimeras can also result in identification of chimeric antigens that protect against pathogen variants that were not included in the

starting population of antigens (for example, protection against strain C by experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library of chimeras/mutants of strains A and B).

- 5           Accordingly, the antigen library immunization approach enables the development of immunogenic polypeptides that can induce immune responses against poorly characterized, newly emerging pathogen variants.

- 10           Sequence reassembly (&/or one or more additional directed evolution methods described herein) can be achieved in many different formats and permutations of formats, as described in further detail below. These formats share some common principles. For example, the targets for modification vary in different applications, as does the property sought to be acquired or improved. Examples of candidate targets for acquisition of a property or improvement in a  
15           property include genes that encode proteins which have immunogenic and/or toxigenic activity when introduced into a host organism.

- The methods use at least two variant forms of a starting target. The variant forms of candidate substrates can show substantial sequence or secondary  
20           structural similarity with each other, but they should also differ in at least one and preferably at least two positions. The initial diversity between forms can be the result of natural variation, e.g., the different variant forms (homologs) are obtained from different individuals or strains of an organism, or constitute related sequences from the same organism (e.g. , allelic variations), or constitute  
25           homologs from different organisms (interspecific variants).

            Alternatively, initial diversity can be induced, e.g., the variant forms can be generated by error-prone transcription, such as an error-prone PCR or use of a polymerase which lacks proof-reading activity (see, Liao (1990) Gene 88:107-

111), of the first variant form, or, by replication of the first form in a mutator strain (mutator host cells are discussed in further detail below, and are generally well known). A mutator strain can include any mutants in any organism impaired in the functions of mismatch repair. These include mutant gene products of mutS, 5 mutT, mutH, mutL, ovrD, dcm, vsr, umuC, umuD, sbcB, recJ, etc. The impairment is achieved by genetic mutation, allelic replacement, selective inhibition by an added reagent such as a small compound or an expressed antisense RNA, or other techniques. Impairment can be of the genes noted, or of homologous genes in any organism. Other methods of generating initial diversity 10 include methods well known to those of skill in the art, including, for example, treatment of a nucleic acid with a chemical or other mutagen, through spontaneous mutation, and by inducing an error-prone repair system (e.g., SOS) in a cell that contains the nucleic acid. The initial diversity between substrates is greatly augmented in subsequent steps of reassembly (&/or one or more additional 15 directed evolution methods described herein) for library generation.

#### Properties involved in immunogenicity

20 Polynucleotide sequences that can positively or negatively affect the immunogenicity of an antigen encoded by the polynucleotide are often scattered throughout the entire antigen gene. Several of these factors are shown diagrammatically herein. By reassembling (&/or subjecting to one or more directed evolution methods described herein) different forms of polynucleotide 25 that encode the antigen using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, followed by selection for those chimeric polynucleotides that encode an antigen that can induce an improved immune response, one can obtain primarily sequences that have a positive influence on antigen immunogenicity. Those sequences that



negatively affect antigen immunogenicity are eliminated. One need not know the particular sequences involved.

5

The present invention provides methods for obtaining polynucleotide sequences that, either directly or indirectly (i.e., through encoding a polypeptide), can modulate an immune response when present on a genetic vaccine vector. In another embodiment, the invention provides methods for optimizing the transport and presentation of antigens. The optimized immunomodulatory polynucleotides obtained using the methods of the invention are particularly suited for use in conjunction with vaccines, including genetic vaccines. One of the advantages of genetic vaccines is that one can incorporate genes encoding immunomodulatory molecules, such as cytokines, costimulatory molecules, and molecules that improve antigen transport and presentation into the genetic vaccine vectors. This provides opportunities to modulate immune responses that are induced against the antigens expressed by the genetic vaccines.

20 **Obtaining components for use in genetic vaccines that are more effective through the creation of a library, the screening of the library, and the use of recombinant nucleic acids that exhibit improved properties.**

In additional embodiments, the present invention provides methods of obtaining components for use in genetic vaccines, including the multicomponent vaccines, that are more effective in conferring a desired immune response property upon a genetic vaccine. The methods involve creating a library of recombinant nucleic acids and screening the library to identify those library members that exhibits an enhanced capacity to confer a desired property upon a

genetic vaccine. Those recombinant nucleic acids that exhibit improved properties can be used as components in a genetic vaccine, either directly as a polynucleotide or as a protein that is obtained by expression of the component nucleic acid.

5

### **Improvement goals**

The properties or characteristics that can be sought to be acquired or improved vary widely, and, of course depend on the choice of substrate. For genetic vaccines, improvement goals include higher titer, more stable expression, improved stability, higher specificity targeting, higher or lower frequency of integration, reduced immunogenicity of the vector or an expression product thereof, increased immunogenicity of the antigen, higher expression of gene products, and the like. Other properties for which optimization is desired include the tailoring of an immune response to be most effective for a particular application. Examples of genetic vaccine components are shown, described &/or referenced herein (including incorporated by reference). Two or more components can be included in a single vector molecule, or each component can be present in a genetic vaccine formulation as a separate molecule.

20

**Sequence reassembly (&/or one or more additional directed evolution methods described herein) can be achieved through different formats which share some common principles**

25

In the methods of the invention, at least two variant forms of a nucleic acid are reassembled (&/or subjected to one or more directed evolution methods described herein) to produce a library of recombinant nucleic acids, which is then screened to identify at least one recombinant component that is optimized for the

particular vaccine property. Often, improvements are achieved after one round of reassembly (&/or one or more additional directed evolution methods described herein) and selection. Sequence reassembly (&/or one or more additional directed evolution methods described herein) can be achieved in many different formats and permutations of formats, as described in further detail below. These formats share some common principles. A family of nucleic acid molecules that have some sequence identity to each other, but differ in the presence of mutations, is typically used as a substrate for reassembly (&/or one or more additional directed evolution methods described herein). In any given cycle, reassembly (&/or one or more additional directed evolution methods described herein) can occur *in vivo* or *in vitro*, intracellularly or extracellularly. Furthermore, diversity resulting from reassembly (&/or one or more additional directed evolution methods described herein) can be augmented in any cycle by applying prior methods of mutagenesis (e.g., error-prone PCR or cassette mutagenesis) to either the substrates or products of reassembly (&/or one or more additional directed evolution methods described herein). In some instances, a new or improved property or characteristic can be achieved after only a single cycle of *in vivo* or *in vitro* reassembly (&/or one or more additional directed evolution methods described herein), as when using different, variant forms of the sequence, as homologs from different individuals or strains of an organism, or related sequences from the same organism, as allelic variations. However, recursive sequence reassembly (&/or one or more additional directed evolution methods described herein), which entails successive cycles of reassembly (&/or one or more additional directed evolution methods described herein), can also be employed to achieve still further improvements in a desired property, or to bring about new (or "distinct") properties, or to generate further molecular diversity.

In a presently preferred embodiment, polynucleotides that encode optimized recombinant antigens are subjected to molecular backcrossing, which

provides a means to breed the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) chimeras/mutants back to a parental or wild-type sequence, while retaining the mutations that are critical to the phenotype that provides the optimized immune responses. In  
5 addition to removing the neutral mutations, molecular backcrossing can also be used to characterize which of the many mutations in an improved variant contribute most to the improved phenotype. This cannot be accomplished in an efficient library fashion by any other method. Backcrossing is performed by reassembling (optionally in combination with other directed evolution methods  
10 described herein) the improved sequence with a large molar excess of the parental sequences.

Stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-  
15 stochastic polynucleotide reassembly is used to obtain the library of  
recombinant nucleic acids, using a variety of substrates to acquire or  
improve various properties for different applications.

#### Creation of Recombinant Libraries

20

The invention involves creating recombinant libraries of polynucleotides that are then screened to identify those library members that exhibit a desired property. The recombinant libraries can be created using any of various methods.

### Initial Diversity Between Substrates

5           The substrate nucleic acids used for the reassembly (&/or one or more additional directed evolution methods described herein) can vary depending upon the particular application. For example, where a polynucleotide that encodes a nucleic acid binding domain or a ligand for a cell-specific receptor is to be optimized, different forms of nucleic acids that encode all or part of the nucleic  
10   acid binding domain or a ligand for a cell-specific receptor are subjected to reassembly (&/or one or more additional directed evolution methods described herein).

          In a presently preferred embodiment, stochastic (e.g. polynucleotide  
15   shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is used to obtain the library of recombinant nucleic acids. stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, which is described herein, can result in optimization of a desired property even in the absence of a detailed understanding of the  
20   mechanism by which the particular property is mediated. The substrates for this modification, or evolution, vary in different applications, as does the property sought to be acquired or improved. Examples of candidate substrates for acquisition of a property or improvement in a property include viral and nonviral vectors used in genetic vaccination, as well as nucleic acids that are involved in  
25   mediating a particular aspect of an immune response. The methods require at least two variant forms of a starting substrate. The variant forms of candidate components can have substantial sequence or secondary structural similarity with each other, but they should also differ in at least two positions. The initial diversity between forms can be the result of natural variation, e.g., the different

variant forms (homologs) are obtained from different individuals or strains of an organism (including geographic variants) or constitute related sequences from the same organism (e.g., allelic variations). Alternatively, the initial diversity can be induced, e.g., the second variant form can be generated by error-prone  
5 transcription, such as an error-prone PCR or use of a polymerase which lacks proof-reading activity (see, Liao (1990) Gene 88:107-111), of the first variant form, or, by replication of the first form in a mutator strain (mutator host cells are discussed in further detail below). The initial diversity between substrates is greatly augmented in subsequent steps of recursive sequence reassembly (&/or  
10 one or more additional directed evolution methods described herein).

Screening or selection after a reassembly (&/or one or more additional directed evolution methods described herein) cycle (screening after *in vitro* and *in vivo* reassembly (&/or one or more additional directed evolution methods  
15 described herein) cycles)

Once one has performed stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to obtain a library of polynucleotides that encode recombinant antigens, the library is  
20 subjected to selection and/or screening to identify those library members that encode antigenic peptides that have improved ability to induce an immune response to the pathogenic agent. Selection and screening of experimentally generated polynucleotides that encode polypeptides having an improved ability to induce an immune response can involve either *in vivo* and *in vitro* methods, but  
25 most often involves a combination of these methods. For example, in a typical embodiment the members of a library of recombinant nucleic acids are picked, either individually or as pools. The clones can be subjected to analysis directly, or can be expressed to produce the corresponding polypeptides. In a presently preferred embodiment, an *in vitro* screen is performed to identify the best

candidate sequences for the *in vivo* studies. Alternatively, the library can be subjected to *in vivo* challenge studies directly. The analyses can employ either the nucleic acids themselves (e.g., as genetic vaccines), or the polypeptides encoded by the nucleic acids. A schematic diagram of a typical strategy shown, described  
5 &/or referenced herein (including incorporated by reference). Both *in vitro* and *in vivo* methods are described in more detail below.

A cycle of reassembly (&/or one or more additional directed evolution methods described herein) is usually followed by at least one cycle of screening  
10 or selection for molecules having a desired property or characteristic. If a cycle of reassembly (&/or one or more additional directed evolution methods described herein) is performed *in vitro*, the products of reassembly (&/or one or more additional directed evolution methods described herein), i.e., recombinant segments, are sometimes introduced into cells before the screening step.  
15 Recombinant segments can also be linked to an appropriate vector or other regulatory sequences before screening.

Alternatively, products of reassembly (&/or one or more additional directed evolution methods described herein) generated *in vitro* are sometimes packaged as  
20 viruses (in viruses- e.g., bacteriophage) before screening. If reassembly (&/or one or more additional directed evolution methods described herein) is performed *in vivo*, product of reassembly (&/or one or more additional directed evolution methods described herein) can sometimes be screened in the cells in which reassembly (&/or one or more additional directed evolution methods described  
25 herein) occurred. In other applications, recombinant segments are extracted from the cells, and optionally packaged as viruses, before screening.

**Component sequences having different roles than the product of reassembly (&/or one or more additional directed evolution methods described herein)**

5       The nature of screening or selection depends on what property or  
characteristic is to be acquired or the property or characteristic for which  
improvement is sought, and many examples are discussed below. It is not usually  
necessary to understand the molecular basis by which particular products of  
reassembly (&/or one or more additional directed evolution methods described  
10       herein) (recombinant segments) have acquired new or improved properties or  
characteristics relative to the starting substrates. For example, a genetic vaccine  
vector can have many component sequences each having a different intended role  
(e.g., coding sequence, regulatory sequences, targeting sequences, stability-  
conferring sequences, immunomodulatory sequences, sequences affecting antigen  
15       presentation, and sequences affecting integration). Each of these component  
sequences can be varied and reassembled (&/or subjected to one or more directed  
evolution methods described herein) simultaneously. Screening/selection can then  
be performed, for example, for recombinant segments that have increased  
episomal maintenance in a target cell without the need to attribute such  
improvement to any of the individual component sequences of the vector.  
20

**Initial screenings in bacterial cells vs. later screening in mammalian cells**

25       Depending on the particular screening protocol used for a desired  
property, initial round(s) of screening can sometimes be performed in bacterial  
cells due to high transfection efficiencies and ease of culture. . However,  
especially for testing of immunogenic activity, test animals are used for library  
expression and screening. Later rounds, and other types of screening which are  
not amenable to screening in bacterial cells, are generally performed (in cells



selected for use in an environment close to that of their intended use) in mammalian cells to optimize recombinant segments for use in an environment close to that of their intended use. Final rounds of screening can be performed in the cell type of intended use (e.g., a human antigen-presenting cell). In some instances, this cell can be obtained from a patient to be treated with a view, for example, to minimizing problems of immunogenicity in this patient. In some methods, use of a genetic vaccine vector in treatment can itself be used as a round of screening. That is, genetic vaccine vectors that are successively taken up and/or expressed by the intended target cells in one patient are recovered from those target cells and used to treat another patient. The genetic vaccine vectors that are recovered from the intended target cells in one patient are enriched for vectors that have evolved, i.e., have been modified by recursive reassembly (&/or one or more additional directed evolution methods described herein), toward improved or new properties or characteristics for specific uptake, immunogenicity, stability, and the like.

#### **Identifying a subpopulation of recombinant segments**

20

The screening or selection step identifies a subpopulation of recombinant segments that have evolved toward acquisition of a new or improved desired property or properties useful in genetic vaccination. Depending on the screen, the recombinant segments can be screened as components of cells, components of viruses or other vectors, or in free form. More than one round of screening or selection can be performed after each round of reassembly (&/or one or more additional directed evolution methods described herein).

25

**The second round of reassembly (&/or one or more additional directed evolution methods described herein)**

If further improvement in a property is desired, at least one and usually a  
5 collection of recombinant segments surviving a first round of screening/selection  
are subject to a further round of reassembly (&/or one or more additional directed  
evolution methods described herein). These recombinant segments can be  
reassembled (&/or subjected to one or more directed evolution methods described  
herein) with each other or with exogenous segments representing the original  
10 substrates or further variants thereof. Again, reassembly (&/or one or more  
additional directed evolution methods described herein) can proceed *in vitro* or *in*  
*vivo*. If the previous screening step identifies desired recombinant segments as  
components of cells, the components can be subjected to further reassembly (&/or  
one or more additional directed evolution methods described herein) *in vivo*, or  
15 can be subjected to further reassembly (&/or one or more additional directed  
evolution methods described herein) *in vitro*, or can be isolated before performing  
a round of *in vitro* reassembly (&/or one or more additional directed evolution  
methods described herein). Conversely, if the previous screening step identifies  
desired recombinant segments in naked form or as components of viruses or other  
20 vectors, these segments can be introduced into cells to perform a round of *in vivo*  
reassembly (&/or one or more additional directed evolution methods described  
herein). The second round of reassembly (&/or one or more additional directed  
evolution methods described herein), irrespective how performed, generates  
further recombinant segments which encompass additional diversity compared to  
25 recombinant segments resulting from previous rounds.

Additional rounds of reassembly (&/or one or more additional directed evolution methods described herein)/screening to sufficiently evolve the recombinant segments

- 5           The second round of reassembly (&/or one or more additional directed evolution methods described herein) can be followed by a further round of screening/selection according to the principles discussed above for the first round. The stringency of screening/selection can be increased between rounds. Also, the nature of the screen and the property being screened for can vary between rounds
- 10   if improvement in more than one property is desired or if acquiring more than one new property is desired.

- Additional rounds of reassembly (&/or one or more additional directed evolution methods described herein) and screening can then be performed until
- 15   the recombinant segments have sufficiently evolved to acquire the desired new or improved property or function.

- The practice of this invention involves the construction of recombinant nucleic acids and the expression of genes in transfected host cells. Molecular
- 20   cloning techniques to achieve these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids such as expression vectors are well-known to persons of skill. General texts which describe molecular biological techniques useful herein, including mutagenesis, include Berger and Kimmel, Guide to Molecular
- 25   Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook") and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing

Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 1998) ("Ausubel").

Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Q - replicase amplification and other RNA polymerase mediated techniques (e. g., NASBA) are found in Berger, Sambrook, and Ausubel, as well as Mullis et al. (1987) U.S. Patent No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis et al. eds) Academic Press Inc. San Diego, CA (1990) (Innis); Antheim & Levinson (October 1, 1990) C&EN 36-47; The Journal Of NIH Research (1991) 3, 81-94; (Kwoh et al. (1989) Proc. Natl. Acad Sci. USA 86, 1173; Guatelli et al. (1990) Proc. Natl. Acad Sci. USA 87, 1874; Lowell et al. (1989) J Clin. Chem 35, 1826; Landegren et al. (1988) Science 241, 1077- 1080; Van Brunt (1990) Biotechnology 8, 291-294; Wu and Wallace (1989) Gene 4, 560; Barringer et al. (1990) Gene 89, 117, and Sooknanan and Malek (1995) Biotechnology 13: 563-564.

Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace et al., U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng et al. (1994) Nature 369: 684-685 and the references therein, in which PCR amplicons of up to 40kb are generated. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. See, Ausubel, Sambrook and Berger, all supra.

Oligonucleotides for use as probes, e.g., in *in vitro* amplification methods, for use as gene probes, or as reassembly targets (e.g., synthetic genes or gene segments) are typically synthesized chemically according to the solid phase

phosphoramidite triester method described by Beaucage and Caruthers (1981) Tetrahedron Letts., 22(20):1859-1862, e.g., using an automated synthesizer, as described in Needham- VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168. Oligonucleotides can also be custom made and ordered from a variety of commercial sources known to persons of skill.

Indeed, essentially any nucleic acid with a known sequence can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (<http://www.genco.com>), ExpressGen Inc. ([www.expressgen.com](http://www.expressgen.com)), Operon Technologies Inc. (Alameda, CA) and many others. Similarly, peptides and antibodies can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, Inc. (<http://www.htibio.com>), BMA Biomedicals Ltd (U.K.), Bio-Synthesis, Inc., and many others.

**Different formats are available for performing reassembly (&/or additional directed evolution methods described herein) and screening/selection which allow for large numbers of mutations in a minimum number of selection cycles and does not require the extensive analysis and computation required by conventional methods.**

A number of different formats are available by which one can create a library of recombinant nucleic acids for screening. In some embodiments, the methods of the invention entail performing reassembly (&/or one or more additional directed evolution methods described herein) and screening or selection to "evolve" individual genes, whole plasmids or viruses, multigene clusters, or even whole genomes (Stemmer (1995) Bio/Technology 13:549-553). Reiterative

cycles of reassembly (&/or one or more additional directed evolution methods described herein) and screening/selection can be performed to further evolve the nucleic acids of interest. Such techniques do not require the extensive analysis and computation required by conventional methods for polypeptide engineering.

5 Reassembly allows the combination of large numbers of mutations in a minimum number of selection cycles, in contrast to traditional, pair wise recombination events (e.g., as occur during sexual replication). Thus, the directed evolution techniques described herein provide particular advantages in that they provide reassembly (optionally in combination with one or more additional directed

10 evolution methods described herein) between any or all of the mutations, thereby providing a very fast way of exploring the manner in which different combinations of mutations can affect a desired result. In some instances, however, structural and/or functional information is available which, although not required for sequence reassembly (&/or one or more additional directed evolution methods

15 described herein), provides opportunities for modification of the technique.

**Four different approaches to improve immunogenic activity as well as broaden specificity: reassembly (optionally in combination with other directed evolution methods described herein) on single gene, sequence comparison of homologous genes, whole genome reassembly, codon modification of polypeptide-encoding genes.**

20

The stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and

25 non-stochastic polynucleotide reassembly methods can involve one or more of at least four different approaches to improve immunogenic activity as well as to broaden specificity. First, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be performed on a single gene. Secondly, several highly homologous genes can be identified by

sequence comparison with known homologous genes. These genes can be synthesized and experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) as a family of homologs, to select recombinants with the desired activity. The experimentally evolved (e.g. by  
5 polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes can be introduced into appropriate host cells, which can include E. coli, yeast, plants, fungi, animal cells, and the like, and those having the desired properties can be identified by the methods described herein. Third, whole genome reassembly can be performed to shuffle genes that can confer a desired property  
10 upon a genetic vaccine (along with other genomic nucleic acids). For whole genome reassembly approaches, it is not even necessary to identify which genes are being experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis). Instead, e.g., bacterial cell or viral genomes are combined and experimentally evolved (e.g. by polynucleotide  
15 reassembly &/or polynucleotide site-saturation mutagenesis) to acquire recombinant nucleic acids that, either itself or through encoding a polypeptide, have enhanced ability to induce an immune response, as measured in any of the assays described herein. Fourth, polypeptide- encoding genes can be codon modified to access mutational diversity not present in any naturally occurring  
20 gene.

**References for formats and examples for sequence reassembly (&/or one or more additional directed evolution methods described herein) and for other**  
25 **methods**

Exemplary formats and examples for polynucleotide reassembly, gene site saturation mutagenesis, interrupted synthesis, and additional directed evolution methods described herein have been described by the present inventors and co-

workers in issued and co-pending applications including USPN 5,965,408 (issued 10-12-99), USPN 5,830,696 (issued 11-03-98), and USPN 5,939,250 (issued 08-17-99).

- 5           Other methods for obtaining libraries of experimentally generated polynucleotides and/or for obtaining diversity in nucleic acids used as the substrates for directed evolution including stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly include, for example, W098/42727; Smith, *Ann. Rev. Genet.* 19: 423-462 (1985);
- 10 Botstein and Shortle, *Science* 229: 1193-1201 (1985); Carter, *Biochem. J* 237: 1-7 (1986); Kunkel, "The efficiency of oligonucleotide directed mutagenesis" in *Nucleic acids & Molecular Biology*, Eckstein and Lilley, eds., Springer Verlag, Berlin (1987)). Included among these methods are oligonucleotide-directed
- 15 mutagenesis (Zoller and Smith, *Nucl. Acids Res.* 10: 6487-6500 (1982), *Methods in Enzymol.* 100: 468-500 (1983), and *Methods in Enzymol.* 154: 329-350 (1987)) phosphothioate-modified DNA mutagenesis (Taylor et al., *Nucl. Acids Res.* 13: 8749-8764 (1985); Taylor et al., *Nucl. Acids Res.* 13: 8765-8787 (1985); Nakamaye and Eckstein, *Nucl. Acids Res.* 14: 9679-9698 (1986); Sayers et al., *Nucl. Acids Res.* 16: 791-802 (1988); Sayers et al., *Nucl. Acids Res.* 16: 803- 814
- 20 (1988)), mutagenesis using uracil-containing templates (Kunkel, *Proc. Nat'l. Acad. Sci. USA* 82: 488- 492 (1985) and Kunkel et al., *Methods in Enzymol.* 154: 367-382)); mutagenesis using gapped duplex DNA (Kramer et al., *Nucl. Acids Res.* 12: 9441-9456 (1984); Kramer and Fritz, *Methods in Enzymol.* 154: 350-367 (1987); Kramer et al., *Nucl. Acids Res.* 16: 7207 (1988)); and Fritz et al., *Nucl.*
- 25 *Acids Res.* 16: 6987-6999 (1988)). Additional suitable methods include point mismatch repair (Kramer et al., *Cell* 38: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter et al., *Nucl. Acids Res.* 13: 4431-4443 (1985); Carter, *Methods in Enzymol.* 154: 382-403 (1987)), deletion mutagenesis (Eghtedarzadeh and Henikoff, *Nucl. Acids Res.* 14: 5115 (1986)), restriction-



- selection and restriction-purification (Wells et al., Phil. Trans. R. Soc. Lond. A 317: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar et al., Science 223: 1299- 1301 (1984); Sakamar and Khorana, Nucl. Acids Res. 14: 6361-6372 (1988); Wells et al., Gene 34: 315- 323 (1985); and Grundström et al.,  
5 Nucl. Acids Res. 13: 3305-3316 (1985). Kits for mutagenesis are commercially available (e.g., Bio-Rad, Amersham International, Anglian Biotechnology).

- For reassembly (&/or one or more additional directed evolution methods**  
10 **described herein) to generate increased diversity relative to the starting**  
**materials, the starting materials must differ from each other in at least two**  
**nucleotide positions.**

- The reassembly procedure starts with at least two substrates that generally  
15 show substantial sequence identity to each other (i.e., at least about 30%, 50%, 70%, 80% or 90% sequence identity), but differ from each other at certain positions. The difference can be any type of mutation, for example, substitutions, insertions and deletions. Often, different segments differ from each other in about 5-20 positions. For reassembly (&/or one or more additional directed evolution  
20 methods described herein) to generate increased diversity relative to the starting materials, the starting materials must differ from each other in at least two nucleotide positions. That is, if there are only two substrates, there should be at least two divergent positions. If there are three substrates, for example, one substrate can differ from the second at a single position, and the second can differ  
25 from the third at a different single position. The starting DNA segments can be natural variants of each other, for example, allelic or species variants. The segments can also be from nonallelic genes showing some degree of structural and usually functional relatedness (e.g., different genes within a superfamily, such as the family of Yersinia V- antigens, for example). The starting DNA segments

can also be induced variants of each other. For example, one DNA segment can be produced by error-prone PCR replication of the other, the nucleic acid can be treated with a chemical or other mutagen, or by substitution of a mutagenic cassette. Induced mutants can also be prepared by propagating one (or both) of the segments in a mutagenic strain, or by inducing an error-prone repair system in the cells.

**The different segments forming the starting materials are related, and might or might not be of similar length**

In these situations, strictly speaking, the second DNA segment is not a single segment but a large family of related segments. The different segments forming the starting materials are often the same length or substantially the same length. However, this need not be the case; for example; one segment can be a subsequence of another. The segments can be present as part of larger molecules, such as vectors, or can be in isolated form.

**The starting DNA segments are reassembled (&/or subjected to one or more directed evolution methods described herein) to generate a library of recombinant DNA segments varying in size which will include full length coding sequences and any essential regulatory**

The starting DNA segments are reassembled (&/or subjected to one or more directed evolution methods described herein) by any of the sequence reassembly (&/or one or more additional directed evolution methods described herein) formats provided herein to generate a diverse library of recombinant DNA segments. Such a library can vary widely in size from having fewer than 10 to

more than  $10^5$ ,  $10^9$ ,  $10^{12}$  or more members. In some embodiments, the starting segments and the recombinant libraries generated will include full-length coding sequences and any essential regulatory sequences, such as a promoter and polyadenylation sequence, required for expression. In other embodiments, the recombinant DNA segments in the library can be inserted into a common vector providing sequences necessary for expression before performing screening/selection.

10 **Using reassembly PCR to assemble multiple segments that have been separately evolved into a full length nucleic acid template such as a gene**

A further technique for recombining mutations in a nucleic acid sequence utilizes "reassembly PCR". This method can be used to assemble multiple segments that have been separately evolved into a full length nucleic acid template such as a gene. This technique is performed when a pool of advantageous mutants is known from previous work or has been identified by screening mutants that may have been created by any mutagenesis technique known in the art, such as PCR mutagenesis, cassette mutagenesis, doped oligo mutagenesis, chemical mutagenesis, or propagation of the DNA template *in vivo* in mutator strains. Boundaries defining segments of a nucleic acid sequence of interest preferably lie in intergenic regions, introns, or areas of a gene not likely to have mutations of interest.

Oligos are synthesized for PCR amplification of segments of the nucleic acid sequence of interest so that the oligos overlap the junctions of two segments by, typically, about 10 to 100 nucleotides

5

Preferably, oligonucleotide primers (oligos) are synthesized for PCR amplification of segments of the nucleic acid sequence of interest, such that the sequences of the oligonucleotides overlap the junctions of two segments. The overlap region is typically about 10 to 100 nucleotides in length. Each of the  
10 segments is amplified with a set of such primers. The PCR products are then "reassembled" according to assembly protocols such as those discussed herein to assemble non-stochastically generated nucleic acid building blocks &/or randomly fragmented genes. In brief, in an assembly protocol the PCR products are first purified away from the primers, by, for example, gel electrophoresis or  
15 size exclusion chromatography. Purified products are mixed together and subjected to about 1-10 cycles of denaturing, reannealing, and extension in the presence of polymerase and deoxynucleoside triphosphates (dNTP's) and appropriate buffer salts in the absence of additional primers ("self-priming"). Subsequent PCR with primers flanking the gene are used to amplify the yield of  
20 the fully reassembled and experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes.

PCR primers are used to introduce variation into the gene of interest and the mutations at sites of interest are screened or selected by sequencing homologues of the nucleic acid sequence

25

In a further embodiment, PCR primers for amplification of segments of the nucleic acid sequence of interest are used to introduce variation into the gene

of interest as follows. Mutations at sites of interest in a nucleic acid sequence are identified by screening or selection, by sequencing homologues of the nucleic acid sequence, and so on.

5

Using oligonucleotide PCR primers (encoding wild type or mutant information) in PCR to generate libraries of full length genes encoding permutations of said info. where the alternative screening or selection process is expensive, cumbersome, or impractical

10

Oligonucleotide PCR primers are then synthesized which encode wild type or mutant information at sites of interest. These primers are then used in PCR mutagenesis to generate libraries of full length genes encoding permutations of wild type and mutant information at the designated positions. This technique is typically advantageous in cases where the screening or selection process is expensive, cumbersome, or impractical relative to the cost of sequencing the genes of mutants of interest and synthesizing mutagenic oligonucleotides.

15

### 2.3. VECTORS USED IN GENETIC VACCINATION

Evolution of genetic vaccines and components by stochastic (e.g.  
5 polynucleotide shuffling & interrupted synthesis) and non-stochastic  
polynucleotide reassembly

The invention provides multicomponent genetic vaccines, and methods of  
10 obtaining genetic vaccine components that improve the capability of the genetic  
vaccine for use in nucleic acid-mediated immunomodulation. A general approach  
for evolution of genetic vaccines and components by stochastic (e.g.  
polynucleotide shuffling & interrupted synthesis) and non-stochastic  
polynucleotide reassembly is shown schematically herein.

15

Including an origin of replication is useful to obtain sufficient quantities of  
the vector prior to administration to a patient, but might be undesirable if  
the vector is designed to integrate into host chromosomal DNA or bind to  
20 host mRNA or DNA.

Broadly speaking, a genetic vaccine vector is an exogenous polynucleotide  
which produces a medically useful phenotypic effect upon the mammalian cell(s)  
25 and organisms into which it is transferred. A vector may or may not have an  
origin of replication. For example, it is useful to include an origin of replication in  
a vector to allow for propagation of the vector in order to obtain sufficient  
quantities of the vector prior to administration to a patient. If the vector is  
designed to integrate into host chromosomal DNA or bind to host mRNA or

DNA, or if replication in the host is otherwise undesirable, the origin of replication can be removed before administration, or an origin can be used that functions in the cells used for vector production but not in the target cells.

However, in certain situations, including some of those discussed herein, it is  
5 desirable that the genetic vaccine vector be capable of replicating in appropriate host cells.

10

**Incorporating nucleic acids that are modified by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly into viral vectors to be used in genetic vaccination**

15

Vectors used in genetic vaccination can be viral or nonviral. Viral vectors are usually introduced into a patient as components of a virus. Illustrative viral vectors into which one can incorporate nucleic acids that are modified by the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-  
20 stochastic polynucleotide reassembly methods of the invention include, for example, adenovirus-based vectors (Cantwell (1996) Blood 88:4676-4683; Ohashi (1997) Proc. Nat'l. Acad. Sci USA 94:1287-1292), Epstein-Barr virus-based vectors (Mazda (1997) J. Immunol. Methods 204:143-151), adenovirus-associated virus vectors, Sindbis virus vectors (Strong (1997) Gene Ther. 4: 624-  
25 627), herpes simplex virus vectors (Kennedy (1997) Brain 120: 1245-1259) and retroviral vectors (Schubert (1997) Curr. Eye Res. 16:656-662).

Techniques for transferring DNA into a cell useful *in vivo* (naked DNA delivered using liposomes fusing to cellular membrane or entering through endocytosis; permeabilize the cells and use DNA binding protein to transport into cell; and bombardment of skin with particles coated with DNA delivered mechanically)

Nonviral vectors, typically dsDNA, can be transferred as naked DNA or associated with a transfer-enhancing vehicle, such as a receptor- recognition protein, liposome, lipoamine, or cationic lipid. This DNA can be transferred into a cell using a variety of techniques well known in the art. For example, naked DNA can be delivered by the use of liposomes which fuse with the cellular membrane or are endocytosed, i.e., by employing ligands attached to the liposome, or attached directly to the DNA, that bind to surface membrane protein receptors of the cell resulting in endocytosis. Alternatively, the cells may be permeabilized to enhance transport of the DNA into the cell, without injuring the host cells. One can use a DNA binding protein, e.g., HBGF-1, known to transport DNA into a cell. Furthermore, DNA can be delivered by bombardment of the skin by gold or other particles coated with DNA which are delivered by mechanical means, e.g., pressure. These procedures for delivering naked DNA to cells are useful *in vivo*. For example, by using liposomes, particularly where the liposome surface carries ligands specific for target cells, or are otherwise preferentially directed to a specific organ, one may provide for the introduction of the DNA into the target cells/organs *in vivo*.



### 2.3.1. VIRAL VECTORS

Structure of viral vectors often consist of a modified viral genome and a coat structure surrounding it, a structure which can be changed in many ways for the viral nucleic acid in a vector designed for genetic vaccination.

Various viral vectors, such as retroviruses, adenoviruses, adenoassociated viruses and herpes viruses, are commonly used in genetic vaccination. They are often made up of two components, a modified viral genome and a coat structure surrounding it (see generally Smith (1995) Annu. Rev. Microbiol. 49, 807-83 8), although sometimes viral vectors are introduced in naked form or coated with proteins other than viral proteins. Most current viral vectors have coat structures similar to a wild type virus. This structure packages and protects the viral nucleic acid and provides the means to bind and enter target cells. In contrast, the viral nucleic acid in a vector designed for genetic vaccination can be changed in many ways. The goals of these changes can be, for example, to enhance or reduce replication of the virus in target cells while maintaining its ability to grow in vector form in available packaging or helper cells, to incorporate new sequences that encode and enable appropriate expression of a gene of interest (e.g., an antigen-encoding gene), and to alter the immunogenicity of the viral vector itself. Viral vector nucleic acids generally comprise two components: essential cis-acting viral sequences for replication and packaging in a helper line and a transcription unit for the exogenous gene. Other viral functions can be expressed in trans in a specific packaging or helper cell line.

### 2.3.1.1. ADENOVIRUSES

#### The normal life cycle and production infection cycle of adenoviruses.

5

Adenoviruses comprise a large class of nonenveloped viruses that contain linear double-stranded DNA. The normal life cycle of the virus does not require dividing cells and involves productive infection in permissive cells during which large amounts of virus accumulate. The productive infection cycle takes about 32-  
10 36 hours in cell culture and comprises two phases, the early phase, prior to viral DNA synthesis, and the late phase, during which structural proteins and viral DNA are synthesized and assembled into virions.

15 In general, adenovirus infections are associated with mild disease in humans.

E3-deletion vectors studied; replication in cultured cells does not require E3 region, allowing insertion of exogenous DNA sequences to yield vectors capable of productive infection and the transient synthesis of relatively large  
20 amounts of encoded protein.

Adenovirus vectors are somewhat larger and more complex than retrovirus or AAV vectors, partly because only a small fraction of the viral genome is removed from most current vectors. If additional genes are removed, they are provided in  
25 trans to produce the vector, which so far has proved difficult. Instead, two general types of adenovirus-based vectors have been studied, E3-deletion and E1-deletion vectors. Some viruses in laboratory stocks of wild-type lack the E3 region and can grow in the absence of helper. This ability does not mean that the E3 gene products are not necessary in the wild, only that replication in cultured cells does

not require them. Deletion of the E3 region allows insertion of exogenous DNA sequences to yield vectors capable of productive infection and the transient synthesis of relatively large amounts of encoded protein.

5

**E1 replacement vectors grown in 293 cells utilized in most gene therapy applications involving adenoviruses.**

Deletion of the E1 region disables the adenovirus, but such vectors can still be grown because there exists an established human cell line (called "293") that contains the E1 region of Ad5 and that constitutively expresses the E1 proteins. Most recent gene-therapy applications involving adenovirus have utilized E1 replacement vectors grown in 293 cells.

15

**Adenovirus vectors capable of efficient episomal gene transfer, easy to grow, can be topically applied to skin for antigen delivery, induction of antigen specific immune responses can be observed, but host response limits duration of expression and ability to repeat dosing in cases with high doses of first generation vectors**

20

The main advantages of adenovirus vectors are that they are capable of efficient episomal gene transfer in a wide range of cells and tissues and that they are easy to grow in large amounts. Adenovirus-based vectors can also be used to deliver antigens after topical application onto the skin, and induction of antigen-specific immune responses can be observed following delivery to the skin (Tang et al. (1997) Nature 388: 729-730). The main disadvantage is that the host response to the virus appears to limit the duration of expression and the ability to repeat dosing, at least with high doses of first- generation vectors.

25

**This invention provides for the first time a phagemid system capable of cloning large DNA inserts of over 10 kilobases and generating ssDNA *in vitro* and *in vivo* corresponding to those large inserts.**

In one embodiment, the directed evolution methods of the invention are used to construct a novel adenovirus-phagemid capable of packaging DNA inserts over 10 kilobases in size. Incorporation of a phage origin in a plasmid using the methods of the invention also generates a novel *in vivo* reassembly or shuffling format capable of evolving whole genomes of viruses, such as the 36 kb family of human adenoviruses. The widely used human adenovirus type 5 (Ad5) has a genome size of 36 kb. It is difficult to shuffle this large genome *in vitro* without creating an excessive number of changes which may cause a high percentage of nonviable recombinant variants. To minimize this problem and achieve whole genome reassembly of Ad5, an adenovirus-phagemid was constructed. The Ad-phagemid has been demonstrated to accept inserts as large as 15 and 24 kilobases and to effectively generate ssDNA of that size. In a further embodiment, larger DNA inserts, as large as 50 to 100 kb are inserted into the Ad-phagemid of the invention; with generation of full length ssDNA corresponding to those large inserts. Generation of such large ssDNA non-stochastically generated nucleic acid building blocks &/or fragments provides a means to evolve, i.e. modify by the recursive reassembly methods (&/or one or more additional recursive directed evolution methods described herein) of the invention, entire viral genomes. Thus, this invention provides for the first time a unique phagemid system capable of cloning large DNA inserts (>10 KB) and generating ssDNA *in vitro* and *in vivo* corresponding to those large inserts.

**In vivo reassembly or shuffling of the genomes of related serotypes of human adenoviruses using system is useful for creation of recombinant adenovirus variants with changes in multiple genes.**

5           The genomes of related serotypes of human adenovirus are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) *in vivo* using this unique phagemid system, as described in International Application No. PCT/US97/17302 (Publ. No. W098/13485). The genomic DNA is first cloned into a phagemid vector, and the resulting plasmid, 10 designated an "Admid," can be used to produce single-stranded (ss) Admid phage by using a helper M13 phage. To achieve *in vivo* reassembly (&/or one or more additional directed evolution methods described herein), ssAdmid phages containing the genome of homologous human adenoviruses are used to perform high multiplicity of infection (MOI) on F<sup>+</sup> MutS E. coli cells. The ssDNA is a 15 better substrate for reassembly (&/or one or more additional directed evolution methods described herein) enzymes such as RecA. The high MOI ensures that the probability of having multiple cross-overs between copies of the infecting ssAdmid DNA is high. The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) adenovirus genome 20 is generated by purification of the double stranded Admid DNA from the infected cells and is introduction into a permissive human cell line to produce the adenovirus library. This genomic reassembly strategy is useful for creation of recombinant adenovirus variants with changes in multiple genes. This allows screening or selection of recombinant variant phenotypes resulting from 25 combinations of variations in multiple genes.

### 2.3.1.2. ADENO-ASSOCIATED VIRUS (AAV)

AAV is a small, simple, nonautonomous virus containing linear single-  
5 stranded DNA. See, Muzycka, Current Topics Microbiol. Immunol. 158, 97- 129  
(1992). The virus requires co-infection with adenovirus or certain other viruses in  
order to replicate. AAV is widespread in the human population, as evidenced by  
antibodies to the virus, but it is not associated with any known disease. AAV  
genome organization is straightforward, comprising only two genes: rep and cap.  
10 The termini of the genome comprises terminal repeats (ITR) sequences of about  
145 nucleotides.

Growth of AAV is cumbersome and helper virus such as adenovirus is often  
15 required.

AAV-based vectors typically contain only the ITR sequences flanking the  
transcription unit of interest. The length of the vector DNA cannot greatly exceed  
the viral genome length of 4680 nucleotides. Currently, growth of AAV vectors is  
20 cumbersome and involves introducing into the host cell not only the vector itself  
but also a plasmid encoding rep and cap to provide helper functions. The helper  
plasmid lacks ITRs and consequently cannot replicate and package. In addition,  
helper virus such as adenovirus is often required.

25

Advantage: long-term expression in nondividing cells.

The potential advantage of AAV vectors is that they appear capable of  
long-term expression in nondividing cells, possibly, though not necessarily,

because the viral DNA integrates. The vectors are structurally simple, and they may therefore provoke less of a host-cell response than adenovirus.

5           **2.3.1.3. PAPILLOMA VIRUS**

Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. Papillomaviruses consist of a single molecule of double-stranded circular DNA about 8,000 bp in size within a  
10 spherical protein coat of 72 capsomeres. Such papillomaviruses are classified by the species they infect (e.g., bovine, human, rabbit) and by type within species. Over 50 distinct human papillomaviruses ("HPV") have been described. See, e.g., Fields Virology (3rd ed., eds. Fields et al., Lippincott-Raven, Philadelphia, 1996).

15

**Cellular tropism for epithelial cells**

Papillomaviruses display a marked degree of cellular tropism for epithelial  
20 cells. Specific viral types have a preference for either cutaneous or mucosal epithelial cells.

**Benign, low-risk, intermediate-risk, and high-risk HPVs.**

25

All papillomaviruses have the capacity to induce cellular proliferation. The most common clinical manifestation of proliferation is the production of benign warts. However, many papillomaviruses have capacity to be oncogenic in some individuals and some papillomaviruses are highly oncogenic. Based on the

pathology of the associated lesions, most human papillomaviruses (HPVs) can be classified in one of four major groups, benign, low-risk, intermediate-risk and high-risk (Fields Virology, (Fields et al., eds., Lippincott- Raven, Philadelphia, 3d ed. 1996); DNA Tumor Viruses: Papilloma in (Encyclopedia of Cancer, Academic Press) Vol. 1, p 520-531). For example, viruses HPV-1, HPV-2, HPV-3, HPV-4, and HPV-27 are associated with benign cutaneous lesions. Viruses HPV-6 and HPV-11 are associated with vulval, penile, and laryngeal warts and are considered low-risk viruses as they are rarely associated with invasive carcinomas. Viruses HPV-16, HPV-18, HPV-31, and HPV-45 are considered high risk virus as they are associated with a high frequency with adeno- and squamous carcinoma of the cervix. Viruses HPV- 5 and HPV-8 are associated with benign cutaneous lesion in a multifactorial disease Epidermodysplasia Verruciformis (EV). Such lesions, however, can progress into squamous cell carcinomas.

15

**HPVs classified for risk based on frequency of cancerous lesions relative to previously classified HPVs.**

These viruses do not fall under one of the four major risk groups. Newly discovered HPVs can classified for risk based on the frequency of cancerous lesions relative to that of HPVs that have already been classified for risk.

HPV vectors can be subjected to iterative cycles of reassembly (&/or one or more additional directed evolution methods described herein) and screening with a view to obtaining vectors with improved properties. Improved properties include increased tissue specificity, altered tissue specificity, increased expression level, prolonged expression, increased episomal copy number, increased or decreased capacity for chromosomal integration, increased uptake capacity, and other properties as discussed herein. The starting materials for reassembling



(optionally in combination with other directed evolution methods described herein) are typically vectors of the kind described above constructed from different strains of human papillomaviruses, or segments or variants of such generated by e.g., error-prone PCR or cassette mutagenesis. The human  
5 papillomaviruses, or at least the E1 and E2 coding regions thereof are preferably human cutaneous papillomaviruses.

#### 2.3.1.4. RETROVIRUSES

10

##### Normal viral life cycle and viral genome organization.

Retroviruses comprise a large class of enveloped viruses that contain single- stranded RNA as the viral genome. During the normal viral life cycle,  
15 viral RNA is reverse- transcribed to yield double-stranded DNA that integrates into the host genome and is expressed over extended periods. As a result, infected cells shed virus continuously without apparent harm to the host cell. The viral genome is small (approximately 10 kb), and its prototypical organization is extremely simple, comprising three genes encoding gag, the group specific  
20 antigens or core proteins; *pol*, the reverse transcriptase; and *env*, the viral envelope protein. The termini of the RNA genome are called long terminal repeats (LTRs) and include promoter and enhancer activities and sequences involved in integration. The genome also includes a sequence required for packaging viral RNA and splice acceptor and donor sites for generation of the  
25 separate envelope mRNA. Most retroviruses can integrate only into replicating cells, although human immunodeficiency virus (HIV) appears to be an exception.

**Providing the missing viral functions to the retrovirus vector and adding/removing additional features to render the vectors more efficacious or reduce the possibility of contamination by helper virus.**

5           Retrovirus vectors are relatively simple, containing the 5' and 3' LTRs, a packaging sequence, and a transcription unit composed of the gene or genes of interest, which is typically an expression cassette. To grow such a vector, one must provide the missing viral functions in trans using a so-called packaging cell line. Such a cell is engineered to contain integrated copies of gag, pol, and env but  
10   to lack a packaging signal so that no helper virus sequences become encapsidated. Additional features added to or removed from the vector and packaging cell line reflect attempts to render the vectors more efficacious or reduce the possibility of contamination by helper virus.

15

**Potentially capable of long-term expression, can be grown in large amounts, but must ensure the absence of helper virus.**

20           For some genetic vaccine applications, retroviral vectors have the advantage of being able integrate in the chromosome and therefore potentially capable of long-term expression. They can be grown in relatively large amounts, but care is needed to ensure the absence of helper virus.

25

### **2.3.2. NON-VIRAL GENETIC VACCINE VECTORS**

Nonviral nucleic acid vectors used in genetic vaccination include plasmids, RNAs, polyamide nucleic acids, and yeast artificial chromosomes (YACs), and the like.

**Vector organization: insertion of enhancer sequence increases transcription.**

- 5           Such vectors typically include an expression cassette for expressing a polypeptide against which an immune response is induced. The promoter in such an expression cassette can be constitutive, cell type-specific, stage-specific, and/or modulatable (e. g., by tetracycline ingestion; tetracycline-responsive promoter). Transcription can be increased by inserting an enhancer sequence into the vector.
- 10          Enhancers are cis-acting sequences, typically between 10 to 300 base pairs in length, that increase transcription by a promoter. Enhancers can effectively increase transcription when either 5' or 3' to the transcription unit. They are also effective if located within an intron or within the coding sequence itself. Typically, viral enhancers are used, including SV40 enhancers, cytomegalovirus
- 15          enhancers, polyoma enhancers, and adenovirus enhancers. Enhancer sequences from mammalian systems are also commonly used, such as the mouse immunoglobulin heavy chain enhancer.

20          **Methods for introduction of nonviral vectors into an animal.**

- Nonviral vectors encoding products useful in gene therapy can be introduced into an animal by means such as lipofection, biolistics, virosomes, liposomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA
- 25          injection, artificial virions, agent-enhanced uptake of DNA, ex vivo transduction. Lipofection is described in e.g., US Patent Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam<sup>TM</sup> and Lipofectin<sup>TM</sup>). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO

91/17424, WO 91/16024. Naked DNA genetic vaccines are described in, for example, US Patent No. 5,589,486.

5    **2.4. MULTICOMPONENT GENETIC VACCINES**

**Use of two or more separate genetic vaccine components for immunization, providing a means for eliciting differentiated responses in different cell types.**

10

The invention provides multicomponent genetic vaccines that are designed to obtain an optimal immune response upon administration to a mammal. In these vaccines, two or more separate genetic vaccine components are used for immunization, preferably in the same formulation. Each component can be  
15 optimized for particular functions that will occur in some cells and not in others, thus providing a means for eliciting differentiated responses in different cell types. When mutually incompatible consequences are derived from use of one plasmid, those activities are separated into different vectors that will have different fates and effects *in vivo*. Genetic vaccines are ideal for the formulation  
20 of several biologically active entities into one preparation. The vectors are preferably all of the same chemical type so there is no incompatibility of this nature, and can all be manufactured by the same chemical and/or biological processes. The vaccine preparation can consist of a defined molar ratio of the separate vector components that can be formulated exactly and repeatedly.

**Developing vector components without knowledge of mechanism by which a particular feature is controlled or property to be modified**

5           Several genetic vaccine vector components that can be used as components of a multicomponent genetic vaccine are described below. The methods of the invention greatly simplify the development of such vector components, because the mechanism by which a particular feature is controlled and the properties of a molecule that, when modified, will enhance that feature,  
10           need not be known. Even in the absence of such knowledge, by carrying out the reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention, one can obtain vector components that are improved for each of the properties listed.

15

**2.4. VECTOR "AR ",DESIGNED TO PROVIDE OPTIMAL ANTIGEN RELEASE**

Genetic vaccine vector component "AR" is designed to provide optimal release of  
20           antigen in a form that will be recognized by antigen presenting cells (APC) and taken up by those cells for efficient intracellular processing and presentation to T helper (T<sub>H</sub>) cells. Cells transfected with AR plasmid can be considered as an antigen factory for APC.

AR plasmids typically have one or more of the following properties, each of  
25           which can be optimized using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention.

**Optimal plasmid binding to and uptake by the chosen antigen expressing cells (e.g., myocytes for intramuscular immunization or epithelial cells for mucosal immunization)**

5           This is a critical property which differentiates AR from other vector components in the multicomponent DNA vaccine. Optimal vector binding to the target cell includes not only the concept of very avid binding and subsequent internalization into target cells, but relative inability to bind to and enter other cells. Optimization of this ratio of desired binding to undesired binding will  
10 significantly increase the number of target cells transfected. This property can be optimized using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly according to the present invention as described herein. For example, variant vector component sequences obtained by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-  
15 stochastic polynucleotide reassembly, combinatorial assembly of vector components, insertion of random oligonucleotide sequences, and the like, can first be selected for those that bind to target cells, after which this population of cells is depleted for those that bind to other cells. Vector components for targeting genetic vaccine vectors to particular cell types, and methods of obtaining improved  
20 targeting, are described in

**(a)           optimal trafficking of the vector DNA to the nucleus.**

Again, the present invention provides methods by which one can obtain genetic vaccine components that are optimal for such properties.

25

**(b)           optimal transcription of the antigen gene(s).**

This can involve, for example, the use of optimized promoters, enhancers, introns, and the like. In a preferred embodiment, cell-specific promoters are used that only allow transcription of the genes when the vector is within the nucleus of the target

cell type. In this case, specificity is derived not only from selective vector entry into target cells.

- (c) **optimal trafficking of mRNA to the cytoplasm and optimal**  
5 **longevity of the mRNA in the cytoplasm.**

To achieve this property, the methods of the invention are used to obtain optimal 3' and 5' non-translated regions of the mRNA.

- (d) **optimal translation of the mRNA.**  
10 Again, the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods are used to obtain optimized recombinant sequences which exhibit optimal ribosome binding and assembly of translational machinery, plus optimal codon preference.

- 15 (e) **optimal antigen structure for efficient uptake by APC.**  
Extracellular antigen is taken up by APC by at least five non-exclusive mechanisms. One mechanism is sampling of the external fluid phase by micropinocytosis and internalization of a vesicle.

20

**Additional mechanistic considerations**

- The first mechanism has, as far as is presently known, no structural requirements for an antigen in the fluid phase and is therefore not relevant to  
25 considerations of designing antigen structure. A second mechanism involves binding of antigen to receptors on the APC surface; such binding occurs according to rules that are only now being studied (these receptors are not immunoglobulin family members and appear to represent several families of proteins and glycoproteins capable of binding different classes of extracellular

proteins/glycoproteins). This type of binding is followed by receptor-mediated internalization, also in a vesicle. Because this mechanism is poorly understood at present, elements of antigen design cannot be incorporated in a rational design process. However, application of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods, an empirical approach of selection of variant DNA molecules most successful at entry into APC, can select for variants that are improved throughout this mechanism.

10           The other three mechanisms all relate to specific antibody recognition of the extracellular antigen. The first mechanism involves immunoglobulin-mediated recognition of the specific antigen via IgG that is bound to Fc receptors on the cell surface. APC such as monocytes, macrophages and dendritic cells can be decorated with surface membrane IgG of diverse specificities. In a primary  
15   response, this mechanism will not be operative. In previously immunized animals, IgG on the surface of APC can specifically bind extracellular antigen and mediate uptake of the bound antigen into an intracellular endosomal compartment. Another mechanism involves binding to clonally-derived surface membrane immunoglobulin which is present on each B cells (IgM in the case of primary B  
20   cells and IgG when the animal has been previously exposed to the antigen). B cells are efficient APC. Extracellular antigen can bind specifically to surface Ig and be internalized and processed in a membrane compartment for presentation on the B cell surface. Finally, extracellular antigen can be recognized by specific soluble immunoglobulin (IgM in the case of a primary immunization and IgG in  
25   the previously immunized animals). Complexing with Ig will elicit binding to the surface of APC (via Fc receptor recognition in the case of IgG) and internalization.



In each of these latter three mechanisms, the extent to which the conformation of the antigen is the same as the recognition specificity of the pre-existing antibody is critical to the efficiency of the process of antigen presentation. Antibodies can recognize linear protein epitopes as well as

5 conformational epitopes determined by the three dimensional structure of the protein antigen. Protective antibodies that will recognize an extracellular virus or bacterial pathogen and by binding to its surface prevent infection or mediate its immune destruction (complement mediated lysis, immune complex formation and phagocytosis) are almost exclusively generated against conformational

10 determinants on the proteins with native structure displayed on the surface of the pathogen. Hence, it is imperative for generation of host protective humoral immunity, to have those naive B cells which bear antibody specific for conformational epitopes present on the pathogen be stimulated by direct contact with T helper cells after intracellular processing of the antigen and presentation of

15 degradation peptides in the context of MHC Class II. This T help will allow selective proliferation of the relevant B cells with consequent mutation of antibody and antigen driven selection for antibodies with increased specificity, as well as antibody class switching.

20 To summarize, optimal uptake of antigen by APC to elicit humoral immunity, as well as specific  $CD4^+$  cytotoxic T cells, requires that the antigen be in native protein conformation (as presented subsequently to the immune system upon natural infection) and recognized by naive B cells bearing the appropriate membrane antibody. Native protein conformation includes appropriate protein

25 folding, glycosylation and any other post- translational modifications necessary for optimal reactivity with the receptors (immunoglobulin and possibly non-immunoglobulin) on APC. In addition to the three dimensional structure of the expressed antigen required for recognition by specific antibody and elicitation of the required immune responses, the structure (and sequence) can be optimized for

- increased protein stability outside the expressing cell, until the time when it is recognized by immune cells, including APCs. The reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention can be used to optimize the antigen structure (and sequence) for subsequent processing after uptake by APC so that intracellular processing results in derivation of the required peptide fragments for presentation on Class I or Class II on APC and desired immune responses.

**(f) optimal partitioning of the nascent antigen into the desired subcellular compartment or compartments.**

- This can be directed by signal and trafficking signals embodied in the antigen sequence. It may be desirable for all of the antigen to be secreted from these cells; alternatively, all or part of the antigen could be directed to be expressed on the cell surface of these factory cells. Signals to direct vesicles containing the antigen to other subcellular compartments for post-translational modifications, including glycosylation, can be embodied in the antigen sequence.

**(g) optimal display of the antigen on the cell surface or optimal release of the antigen from the cells.**

- A variation on items (f) and (g) is to design the expression of the antigen within the cytoplasm of the factory cell followed by lysis of that cell to release soluble antigen. Cell death can be engineered by expression on the same genetic vaccine vector of an intracellular protein that will elicit apoptosis. In this case, the timing of cell death is balanced with the need for the cell to produce antigen, as well as the potential deleterious effect of killing some cells in a designed process.

In combination, items (a) -(h) lead to a variety of scenarios for the optimizing the longevity and extent of antigen expression. It is not always desirable that the antigen be expressed for the longest time at the highest level. In

certain clinical applications, it will be important to have antigen expression that is short time-low expression, short time-high expression, long time-low expression, long time-high expression or somewhere in between.

5 Plasmid AR can be designed to express one or more variants of a single antigen gene or several quite different targets for immunization. Methods for obtaining optimized antigens for use in genetic vaccines are described herein. Multiple antigens can be expressed from a monocistronic or multicistronic form of the vector.

10

#### **2.4.2. VECTOR COMPONENTS "CTL-DC", "CTL-LC" AND "CTL-MM", DESIGNED FOR OPTIMAL PRODUCTION OF CTLs**

15 Genetic vector components "CTL-DC", "CTL-LC" and "CTL-MM" are designed to direct optimal production of cytotoxic CD8<sup>+</sup> lymphocytes (CTLs) by dendritic cells (CTL-DC), Langerhan's cells (CTL-LC), and monocytes and macrophages (CTL-MM) These vector components direct presentation of optimal antigen fragments in association with MHC Class I, thereby ensuring maximal  
20 cytotoxic T cell immune responses. Cells transfected with CTL vector components can be considered as the direct activators of this arm of specific immunity that is usually critically important for protection against viral diseases.

CTL vector components are typically designed to have one or more of the  
25 following properties, each of which can be optimized using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention:

(a) **optimal vector binding to, and uptake by, the chosen antigen presenting cells (e.g., dendritic cells, monocytes/macrophages, Langerhan's cells).**

This is a critical property to differentiate CTL series vectors from other vectors in the multicomponent DNA vaccine. CTL series vectors preferably do not bind to or enter cells that are chosen to be the extracellular antigen expression host via AR vectors. This separation of functions is critical, as the intracellular fate and trafficking of antigen destined for stimulation of immune cells after release from an antigen expressing cell is quite different than the fate of antigen destined to be presented on the cell surface in association with MHC Class I. In the former case, antigen is directed via a signal secretion sequence to be delivered intact to the lumen of the rough endoplasmic reticulum (RER) and then secreted. In the latter case, antigen is directed to remain in the cytoplasm and there be degraded into peptide fragments by the proteasomal system followed by delivery to the lumen of the RER for association with MHC Class I. These complexes of peptide and MHC Class I are then delivered to the cell surface for specific interaction with CD8<sup>+</sup> cytotoxic T cells. Vector components, and methods for obtaining optimized vector components, that are optimized for targeting to desired cell types are described in

20

**Optimizing transcription of the antigen gene(s)**

This can be accomplished by optimizing promoters, enhancers, introns, and the like, as discussed herein. Cell specific promoters are valuable in such vectors as an additional level of selectivity.

25

(b) **optimal longevity of the mRNA.**

Optimal 3' and 5' non-translated regions of the mRNA can be obtained using the methods of the invention.

**(c) optimal translation of the mRNA.**

Again, the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly and selection methods of the invention can be used to obtain polynucleotide sequences for optimal ribosome binding and assembly of translational machinery, as well as optimal codon preference.

**(d) optimal protein conformation.**

In this case, the optimal protein conformation yields appropriate cytoplasmic proteolysis and production of the correct peptides for presentation on MHC Class I and elicitation of the desired specific CTL responses, rather than a conformation that will interact with specific antibody or other receptors on the surface of APC.

**(e) optimal proteolysis to generate the correct peptides.**

The order of specific proteolytic cleavages will depend on the nature of protein folding and the nature of proteases either in the cytoplasm or in the proteasome.

**(f) optimal transport of the antigen peptides across the endoplasmic reticulum membrane to be delivered into the RER lumen.**

This may be mediated by recognition of the peptides by TAP proteins or by other membrane transporters.

**(h) optimal association of the peptides with the Class I- 2 microglobulin complex and trafficking to the cell surface via the secretory pathway.**

**(i) optimal display of the MHC-peptide complex with associated accessory molecules for recognition by specific CTL.**

Vector CTL can be designed to express one or more variants of a single antigen gene or several different targets for immunization. Multiple optimized antigens can be expressed from a monocistronic or multicistronic form of the vector.

### 2.4.3. VECTORS "M" DESIGNED FOR OPTIMAL RELEASE OF IMMUNE MODULATORS

5

Vectors "M" are designed to direct optimal release of immune modulators, such as cytokines and other growth factors, from target cells. Target cells can be either the predominant cell type in the immunized tissue or immune cells such as dendritic cells (M-DC), Langerhan's cells (M-LC), monocytes & macrophages (M-MM)". These vectors direct simultaneous expression of optimal levels of several immune cell "modulators" (cytokines, growth factors, and the like) such that the immune response is of the desired type, or combination of types, and of the desired level. Cells transfected with M vectors can be considered as the directors of the nature of the vaccine immune response (CTL vs  $T_H1$  vs  $T_H2$  vs NK cell, etc.) and its magnitude. The properties of these vectors reflect the nature of the cell in which the vectors are designed to operate. For example, the vectors are designed to bind to and enter the desired cell type, and/or can have cell-specific regulated promoters that drive transcription in the desired cell type. The vectors can also be engineered to direct maximal synthesis and release of the cell modulator proteins from the target cells in the desired ratio.

"M" genetic vaccine vectors are typically designed to have one or more of the following properties, each of which can be optimized using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention:

(a) **optimal vector binding to and uptake by the chosen modulator expressing cell.**

Suitable expressing cells include, for example, muscle cells, epithelial cells or  
5 other dominant (by number) cell types in the target tissue, antigen presenting cells  
(e.g. dendritic cells, monocytes/macrophages, Langerhans cells). This is a critical  
property which differentiates M series vectors from those designed to bind to and  
enter other cells.

10 (b) **optimal transcription of the immune modulator gene(s).**

Again, promoters, enhancers, introns, and the like can be optimized according to  
the methods of the invention. Cell specific promoters are very valuable here as an  
additional level of selectivity.

15 (c) **optimal longevity of the mRNA.**

Optimal 3' and 5' non-translated regions of the mRNA can be obtained using the  
methods of the invention.

(d) **optimal translation of the mRNA.**

20 Again, the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and  
non-stochastic polynucleotide reassembly and selection methods of the invention  
can be used to obtain polynucleotide sequences for optimal ribosome binding and  
assembly of translational machinery, as well as optimal codon preference.

25 (e) **optimal trafficking of the modulator into the lumen of the RER  
(via a signal secretion sequence).**

An alternative strategy for modulation of the immune response uses membrane  
anchored modulators rather than secretion of soluble modulator. Anchored

modulator can be retained on the surface of the synthesizing cell by, for example, a hydrophobic tail and phosphoinositol glycan linkage.

(f) **optimal protein conformation for each modulator.**

- 5 In this case, the optimal protein conformation is that which allows extracellular modulator and/or cell membrane anchored modulator to interact with the relevant receptor.

(g) **the ratio of modulators and their type can be determined**

10 **empirically.**

- One will test sets of modulators that are known to work in concert to direct the immune response in the direction of a  $T_H$  response (e.g., production of IL-2 and/or IFN ) or  $T_H2$  response (e.g., IL-4, IL-5, IL-13), for example. Vector M can be designed to express one or more modulators. Optimized immunomodulators, and  
15 methods for obtaining optimized immunomodulators, are described herein. These optimized immunomodulatory sequences are particularly suitable for use as components of the multicomponent genetic vaccines of the invention. Multiple modulators can be expressed from a monocistronic or multicistronic form of the vector.

20

**2.4.4. VECTORS "CK", DESIGNED TO DIRECT RELEASE OF  
CHEMOKINES**

25

Genetic vaccine vectors designated "CK" are designed to direct optimal release of chemokines from target cells. Target cells can be either the predominant cell type in the immunized tissue, or can be immune cells such as dendritic cells (CK-DC), Langerhan's cells (CK-LC), or monocytes and macrophages (CK-MM).



These vectors typically direct simultaneous expression of optimal levels of several chemokines such that the recruitment of immune cells to the site of immunization is optimal. Cells transfected with CK vectors can be considered as the traffic police, regulating the immune cells critical for the vaccine immune response. The properties of these vectors reflect the nature of the cell in which the vectors are designed to operate. For example, the vectors are designed to bind to and enter the desired cell type, and/or can have cell-specific regulated promoters that drive transcription in the desired cell type. The vectors are also engineered to direct maximal synthesis and release of the chemokines from the target cells in the desired ratio. Genetic vaccine components, and methods for obtaining components, that provide optimal release of chemokines are described herein.

CK vectors are typically designed to have one or more of the following properties, each of which can be optimized using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention:

20

**(a) optimal vector binding to and uptake by the chosen chemokine expressing cell.**

Suitable cells include, for example, muscle cells, epithelial cells, or cell types that are dominant (by number) in the particular tissue of interest. Also suitable are antigen presenting cells (e.g. dendritic cells, monocytes and macrophages, Langerhans cells). This is a critical property which differentiates CK series vectors from those designed to bind to and enter other cells.

**(b) optimal transcription of the chemokine gene(s).**

Again, promoters, enhancers, introns, and the like can be optimized according to the methods of the invention.

5 Cell specific promoters are very valuable here as an additional level of selectivity.

**(c) optimal longevity of the mRNA.**

Optimal 3' and 5' non-translated regions of the mRNA can be obtained using the methods of the invention.

10

**(d) optimal translation of the mRNA.**

Again, the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly and selection methods of the invention can be used to obtain polynucleotide sequences for optimal ribosome binding and assembly of translational machinery, as well as optimal codon preference.

15

**(e) optimal trafficking of the chemokine into the lumen of the RER (via a signal secretion sequence).**

An alternative strategy for modulation of the immune response via recruitment of cells will use membrane anchored chemokine rather than secretion of soluble chemokine. Anchored chemokine will be retained on the surface of the synthesizing cell by a hydrophobic tail and phosphoinositol glycan linkage.

20

**(f) optimal protein conformation for each chemokine.**

In this case, the optimal protein conformation is that which allows extracellular chemokine/cell membrane anchored chemokine to interact with the relevant receptor.

25

(g) the ratio of diverse chemokines can be determined empirically.

One can test sets of chemokines that are known to work in concert to direct recruitment of CTL, T<sub>H</sub> cells, B cells, monocytes/macrophages, eosinophils, and/or neutrophils as appropriate.

Vector CK can be designed to express one or more chemokines. Multiple chemokines can be expressed from a monocistronic or multicistronic form of the vector.

#### 2.4.5. OTHER VECTORS

Genetic vaccines which contain one or more additional component vector moieties are also provided by the invention. For example, the genetic vaccine can include a vector that is designed to specifically enter dendritic cells and Langerhans cells, and will migrate to the draining lymph nodes.

This vector is designed to provide for expression of the target antigen(s), as well as a cocktail of cytokines and chemokines relevant to elicitation of the desired immune response in the node

Depending on the clinical goals and nature of the antigen, the vector can be optimized for relatively long lived expression of the target antigen so that stimulation of the immune system is prolonged at the node. Another example is a vector that specifically modulates MHC expression in B cells. Such vectors are designed to specifically bind to and enter B cells, cells either resident in the injection site or attracted into the site. Within the B cell, this vector directs the association of antigen peptides derived from specific uptake of antigen into the

endocytic compartment of the cell to either association with Class I or Class II, hence directing the elicitation of specific immunity via CD4<sup>+</sup> T helper cells or CD8<sup>+</sup> cytotoxic lymphocytes. Numerous means exist for this intracellular direction of the fate of processed peptide that are discussed herein.

5

Examples of molecules that direct Class I presentation include tapasin, TAP-1 and TAP-2 (Koopman et al. (1997) Curr. Opin. Immunol. 9: 80-88), and those affecting Class II presentation include, for example, endosomal/lysosomal proteases (Peters (1997) Curr. Opin. Immunol. 9: 89-96). Genetic vaccine components, and methods for obtaining components, that provide optimized Class I presentation are described herein. An optimal DNA vaccine could, for example, combine an AR vector (antigen release), a CTL-DC vector (CTL activation via dendritic cell presentation of antigen peptide on MHC Class I), an M-MM vector for release of IL- 12 and IFN $\gamma$  from resident tissue macrophages, and a CK vector for recruitment of T<sub>H</sub> cells into the immunization site.

20

**Directed evolution aid the following DNA vaccination goals**

DNA vaccination can be used for diverse goals that can include the following, among others:

- 25
- stimulation of a CTL response and/or humoral response ready to react rapidly and aggressively against an invading bacterial or viral pathogen at some time in the distant future
  - a continuous but non-aggressive response to prevent inappropriate responses to allergens

- a continuous non-aggressive and tolerization of immunity to an autoantigen in autoimmune disease
- elicitation of an aggressive CTL response as rapidly as possible against tumor cell antigens
- 5 • redirection of the immune response away from a strong but inappropriate immune response to an on-going chronic infection in the direction of desired responses to clear the pathogen and/or prevent pathology.

These goals cannot always be met by the format of a single vector DNA vaccine, particularly wherein competing goals are embodied within one DNA sequence. A multicomponent format allows the generation of a portfolio of DNA vaccine vectors, some of which will be reconstructed on each occasion (e.g., those vectors containing antigen) while others will be used as well characterized and understood reagents for numerous different clinical applications (e.g., the same chemokine-expressing vector can be used in different situations).

## 2.5. SCREENING METHODS

20 Screening assay varies depending of property for which improvement is sought

Recombinant nucleic acid libraries that are obtained by the methods described herein are screened to identify those DNA segments that have a property which is desirable for genetic vaccination. The particular screening assay employed will vary, as described below, depending on the particular property for which improvement is sought. Typically, the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acid library is introduced into cells prior to screening. If the stochastic

(e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly format employed is an *in vivo* format, the library of recombinant DNA segments generated already exists in a cell. If the sequence reassembly (&/or one or more additional directed evolution methods described  
5 herein) is performed *in vitro*, the recombinant library is preferably introduced into the desired cell type before screening/selection. The members of the recombinant library can be linked to an episome or virus before introduction or can be introduced directly.

10

### Cell types

A wide variety of cell types can be used as a recipient of evolved genes. Cells of particular interest include many bacterial cell types that are used to  
15 deliver vaccines or vaccine antigens (Courvalin et al.(1995) C. R. Acad. Sci. 11118: 1207- 12), both gram- negative and gram-positive, such as salmonella (Attridge et al. (1997) Vaccine 15: 155-62), clostridium. (Fox et al. (1996) Gene Ther. 3: 173-8), lactobacillus, shigella (Sizemore et al. (1995) Science 270: 299-302), E. coli, streptococcus (Oggioni and Pozzi (1996) Gene 169: 85-90), as well  
20 as mammalian cells, including human cells. In some embodiments of the invention, the library is amplified in a first host, and is then recovered from that host and introduced to a second host more amenable to expression, selection, or screening, or any other desirable parameter. The manner in which the library is introduced into the cell type depends on the DNA-uptake characteristics of the  
25 cell type, e.g., having viral receptors, being capable of conjugation, or being naturally competent. If the cell type is unsusceptible to natural and chemical-induced competence, but susceptible to electroporation, one would usually employ electroporation. If the cell type is unsusceptible to electroporation as well, one can employ biolistics. The biolistic PDS-1000 Gene Gun (Biorad, Hercules,

CA) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells.

5    **Competent or Potentially Competent Tissue**

          The process is applicable to a wide range of tissues, including plants, bacteria, fungi, algae, intact animal tissues, tissue culture cells, and animal embryos. One can employ electronic pulse delivery, which is essentially a mild  
10    electroporation format for live tissues in animals and patients (Zhao, Advanced Drug Delivery Reviews 17:257-262 (1995)). Novel methods for making cells competent are described in International Patent Application PCT/US97/04494 (Publ. No. W097/35957). After introduction of the library of recombinant DNA genes, the cells are optionally propagated to allow expression of genes to occur.

15

**Identifying cells that contain a vector through inclusion of a selectable marker gene**

20           In many assays, a means for identifying cells that contain a particular vector is necessary. Genetic vaccine vectors of all kinds can include a selectable marker gene. Under selective conditions, only those cells that express the selectable marker will survive.

25

**Examples of Selectable Marker Genes**

          Examples of suitable markers include, the dihydrofolate reductase gene (DHFR), the thymidine kinase gene (TK), or prokaryotic genes conferring drug

resistance, *gpt* (xanthine- guanine phosphoribosyltransferase, which can be selected for with mycophenolic acid; *neo* (neomycin phosphotransferase), which can be selected for with G418, hygromycin, or puromycin; and DHFR (dihydrofolate reductase), which can be selected for with methotrexate (Mulligan & #0000; Southern & Berg (1982) J Mol. Appl. Genet. 1: 327).

**Identifying cells that contain a vector through inclusion of a screenable marker gene**

As an alternative to, or in addition to, a selectable marker, a genetic vaccine vector can include a screenable marker which, when expressed, confers upon a cell containing the vector a readily identifiable phenotype. For example, gene that encodes a cell surface antigen that is not normally present on the host cell is suitable. The detection means can be, for example, an antibody or other ligand which specifically binds to the cell surface antigen. Examples of suitable cell surface antigens include any CD (cluster of differentiation) antigen (CD1 to CD163) from a species other than that of the host cell which is not recognized by host-specific antibodies. Other examples include green fluorescent protein (GFP, see, e.g., Chalfie et al. (1994) Science 263:802-805; Crameri et al. (1996) Nature Biotechnol. 14: 315-319; Chalfie et al. (1995) Photochem. Photobiol. 62:651-656; Olson et al. (1995) J Cell. Biol. 130:639-650) and related antigens, several of which are commercially available.



### 2.5.1. SCREENING FOR VECTOR LONGEVITY OR TRANSLOCATION TO DESIRED TISSUE

5           For certain applications, it is desirable to identify those vectors with the greatest longevity as DNA, or to identify vectors which end up in tissues distant from the injection site. This can be accomplished by administering to an animal a population of recombinant genetic vaccine vectors by the chosen route of administration and, at various times thereafter excise the target tissue and recover  
10   vector from the tissue by standard molecular biology procedures. The recovered vector molecules can be amplified in, for example, *E. coli* and/ or by PCR *in vitro*. The PCR amplification can involve further polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein), after which the derived selected  
15   population used for readministration to animals and further improvement of the vector. After several rounds of this procedure, the selected vectors can be tested for their capacity to express the antigen in the correct conformation under the same conditions as the vector was selected *in vivo*.

#### 20   Methods for *in vitro* identification of cells expressing the desired antigen

          Because antigen expression is not part of the selection or screening process described above, not all vectors obtained are capable of expressing the desired antigen. To overcome this drawback, the invention provides methods for  
25   identifying those vectors in a genetic vaccine population that exhibit not only the desired tissue localization and longevity of DNA integrity *in vivo*, but retention of maximal antigen expression (or expression of other genes such as cytokines, chemokines, cell surface accessory molecules, MHC, and the like).

The methods involve *in vitro* identification of cells which express the desired molecule using cells purified from the tissue of choice, under conditions that allow recovery of very small numbers of cells and quantitative selection of those with different levels of antigen expression as desired.

5

Two embodiments of the invention are described, each of which uses a library of genetic vaccine vectors as the starting point. The goal of each method is to identify those vectors that exhibit the desired biological properties *in vivo*. The recombinant library represents a population of vectors that differ in known ways (e.g., a combinatorial vector library of different functional modules), or has randomly generated diversity generated either by insertion of random nucleotide stretches, or has been experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) *in vitro* to introduce low level mutations across all or part of the vector.

15

#### 2.5.1.1. SELECTION FOR EXPRESSION OF CELL SURFACE- LOCALIZED ANTIGEN

20 In a first embodiment, the invention method involves selection for expression of cell surface-localized antigen. The antigen gene is engineered in the vaccine vector library such that it has a region of amino acids which is targeted to the cell membrane. For example, the region can encode a hydrophobic stretch of C-terminal amino acids which signals the attachment of a phosphoinositol-glycan (PIG) terminus on the expressed protein and directs the protein to be expressed on  
25 the surface of the transfected cell. With an antigen that is naturally a soluble protein, this method will likely not affect the three dimensional folding of the protein in this engineered fusion with a new C-terminus. With an antigen that is naturally a transmembrane protein (e.g., a surface membrane protein on

pathogenic viruses, bacteria, protozoa or tumor cells) there are at least two possibilities. First, the extracellular domain can be engineered to be in fusion with the C- terminal sequence for signaling PIG-linkage. Second, the protein can be expressed *in toto* relying on the signaling of the host cell to direct it efficiently to the cell surface. In a minority of cases, the antigen for expression will have an endogenous PIG terminal linkage (e.g., some antigens of pathogenic protozoa).

#### **Collection, purification, identification and separation of target cells**

10

The vector library is delivered *in vivo* and, after a suitable interval of time tissue and/or cells from diverse target sites in the animal are collected. Cells can be purified from the tissue using standard cell biological procedures, including the use of cell specific surface reactive monoclonal antibodies as affinity reagents. It is relatively facile to purify isolated epithelial cells from mucosal sites where epithelium may have been inoculated or myoblasts from muscle. In some embodiments, minimal physical purification is performed prior to analysis. It is sometimes desirable to identify and separate specific cell populations from various tissues, such as spleen, liver, bone marrow, lymph node, and blood. Blood cells can be fractionated readily by FACS to separate B cells, CD4<sup>+</sup> or CD8<sup>+</sup> T cells, dendritic cells, Langerhans cells, monocytes, and the like, using diverse fluorescent monoclonal antibody reagents.

#### **Identification and purification of cells expressing the antigen**

Those cells expressing the antigen can be identified with a fluorescent monoclonal antibody specific for the C-terminal sequence on PIG-linked forms of the surface antigen. FACS analysis allows quantitative assessment of the level of

expression of the correct form of the antigen on the cell population. Cells expressing the maximal level of antigen are sorted and standard molecular biology methods used to recover the plasmid DNA vaccine vector that conferred this reactivity. An alternative procedure that allows purification of all those cells  
5 expressing the antigen (and that may be useful prior to loading onto a cell sorter since antigen expressing cells may be a very small minority population), is to rosette or pan-purify the cells expressing surface antigen. Rosettes can be formed between antigen expressing cells and erythrocytes bearing covalently coupled antibody to the relevant antigen. These are readily purified by unit gravity  
10 sedimentation. Panning of the cell population over petri dishes bearing immobilized monoclonal antibody specific for the relevant antigen can also be used to remove unwanted cells.

Cells expressing the required conformational structure of the target antigen  
15 can be identified using specific conformationally-dependent monoclonal antibodies that are known to react specifically with the same structure as expressed on the target pathogen.

20 Using several monoclonal antibodies in the selection process to minimize the possibility of an antigen which reacts with high affinity to the diagnostic antibody but does not yield the correct conformation

Because one monoclonal antibody cannot define all aspects of correct  
25 folding of the target antigen, one can minimize the possibility of an antigen which reacts with high affinity to the diagnostic antibody but does not yield the correct conformation as defined by that in which the antigen is found on the surface of the target pathogen or as secreted from the target pathogen. One way to minimize this possibility is to use several monoclonal antibodies, each known to react with

different conformational epitopes in the correctly folded protein, in the selection process. This can be achieved by secondary FACS sorting for example.

5 The enriched plasmid population that successfully expressed sufficient of the antigen in the correct body site for the desired time is then used as the starting population for another round of selection, incorporating gene reassembling (optionally in combination with other directed evolution methods described herein) to expand the diversity. In this manner, one recovers the desired biological activity encoded by plasmid from tissues in DNA vaccine-immunized animals.

10

This method can also provide the best *in vivo* selected vectors that express immune accessory molecules that one may wish to incorporate into DNA vaccine constructs. For example, if it is desired to express the accessory protein B7.1 or B7.2 in antigen-presenting-cells (APC) (to promote successful presentation of antigen to T cells) one can sort APC isolated from different tissues (at or different to the inoculation site) using commercially available monoclonal antibodies that recognize functional B7 proteins.

20

#### 2.5.1.2. SELECTION FOR EXPRESSION OF SECRETED ANTIGEN/CYTOKINE/CHEMOKINE

Select vectors that are optimal in inducing secretion of soluble proteins that can affect the qualitative and quantitative nature of an elicited immune response *in vivo*

25

The invention also provides methods to identify plasmids in a genetic vaccine vector population that are optimal in secretion of soluble proteins that can affect the qualitative and quantitative nature of an elicited immune response. For

example, the methods are useful for selecting vectors that are optimal for secretion of particular cytokines, growth factors and chemokines. The goal of the selection is to determine which particular combinations of cytokines, chemokines and growth factors, in combination with different promoters, enhancers, polyA  
5 tracts, introns, and the like, elicits the required immune response *in vivo*.

Genes encoding the polypeptides are typically present in the vaccine vector library in combination with optimal signal secretion sequences (proteins are  
10 secreted from the cells.)

Combinations of the genes for the soluble proteins of interest can be present in the vectors; transcription can be either from a single promoter, or the genes can be placed in multicistronic arrangements. Typically, the genes encoding  
15 the polypeptides are present in the vaccine vector library in combination with optimal signal secretion sequences, such that the expressed proteins are secreted from the cells.

20 Generating vectors capable of secreting different combinations of soluble factors *in vitro* and capable of expressing those factors for desired lengths of time.

The first step in these methods is to generate vectors that are capable of  
25 secreting high (or in some case low) levels of different combinations of soluble factors *in vitro* and that will express those factors for a short or long time as desired. This method allows one to select for and retain an inventory of plasmids which can be characterized by known patterns of soluble protein expression in known tissues for a known time. These vectors can then be tested individually for

*in vivo* efficacy, after being placed in combination with the genetic vaccine antigen in an appropriate expression construct.

**5    Delivery of vector library and subsequent collection, testing, and purification using FACS sorting, affinity panning, rosetting, or magnetic bead separation to separate cell populations prior to identification**

10        The vector library is delivered to a test animal and, after a chosen interval of time, tissue and/or cells from diverse sites on the animal are collected. Cells are purified from the tissue using standard cell biological procedures, which often include the use of cell specific surface reactive monoclonal antibodies as affinity reagents. As is the case for cell surface antigens described above, physical purification of separate cell populations can be performed prior to identification  
15        of cells which express the desired protein. For these studies, the target cells for expression of cytokines will most usually be APC or B cells or T cells rather than muscle cells or epithelial cells. In such cases FACS sorting by established methods will be preferred to separate the different cell types. The different cell types described above may also be separated into relatively pure fractions using  
20        affinity panning, rosetting or magnetic bead separation with panels of existing monoclonal antibodies known to define the surface membrane phenotype of murine immune cells.

**Identifying and selecting purified cells through visual inspection or flow cytometry for use in another round of selection incorporating gene reassembling (optionally in combination with other directed evolution methods described herein) to expand the diversity**

Purified cells are plated onto agar plates under conditions that maintain cell viability. Cells expressing the required conformational structure of the target antigen are identified using conformationally-dependent monoclonal antibodies that are known to react specifically with the same structure as expressed on the target pathogen. Release of the relevant soluble protein from the cells is detected by incubation with monoclonal antibody, followed by a secondary reagent that gives a macroscopic signal (gold deposition, color development, fluorescence, luminescence). Cells expressing the maximal level of antigen can be identified by visual inspection, the cell or cell colony picked and standard molecular biology methods used to recover the plasmid DNA vaccine vector that conferred this reactivity. Alternatively, flow cytometry can be used to identify and select cells harboring plasmids that induce high levels of gene expression. The enriched plasmid population that successfully expressed sufficient of the soluble factor in the correct body site for the desired time is then used as the starting population for another round of selection, incorporating gene reassembling (optionally in combination with other directed evolution methods described herein) to expand the diversity, if further improvement is desired. In this manner, one recovers the desired biological activity encoded by plasmid from tissues in DNA vaccine-immunized animals.



Using monoclonal antibody to confirm that the initial results from screening still hold when several conformational epitopes are probed

- 5           Several monoclonal antibodies, each known to react with different conformational epitopes in the correctly folded cytokine, chemokine or growth factor, can be used to confirm that the initial results from screening with one monoclonal antibody reagent still hold when several conformational epitopes are probed. In some cases the primary probe for functional cytokine released from the
- 10   cell/cell colony in agar could be a soluble domain of the cognate receptor.

#### 2.5.2. FLOW CYTOMETRY

- 15   Most of the vector module libraries can be assayed by flow cytometry to select individual human tissue culture cells that contain the experimentally generated nucleic acid sequences that have the greatest improvement in the desired property

- 20           Flow cytometry provides a means to efficiently analyze the functional properties of millions of individual cells. The cells are passed through an illumination zone, where they are hit by a laser beam; the scattered light and fluorescence is analyzed by computer-linked detectors. Flow cytometry provides several advantages over other methods of analyzing cell populations. Thousands
- 25   of cells can be analyzed per second, with a high degree of accuracy and sensitivity. Gating of cell populations allows multiparameter analysis of each sample. Cell size, viability, and morphology can be analyzed without the need for staining. When dyes and labeled antibodies are used, one can analyze DNA content, cell surface and intracytoplasmic proteins, and identify cell type,

activation state, cell cycle stage, and detect apoptosis. Up to four colors (thus, four separate antigens stained with different fluorescent labels) and light scatter characteristics can be analyzed simultaneously (four colors requires two-laser instrument; one-laser instrument can analyze three colors). The expression levels of several genes can be analyzed simultaneously, and importantly, flow cytometry-based cell sorting ("FACS sorting") allows selection of cells with desired phenotypes. Most of the vector module libraries, including the promoter, enhancer, intron, episomal origin of replication, expression level aspect of antigen, bacterial origin and bacterial marker, can be assayed by flow cytometry to select individual human tissue culture cells that contain the reassembled (&/or subjected to one or more directed evolution methods described herein) nucleic acid sequences that have the greatest improvement in the desired property. Typically the selection is for high level expression of a surface antigen or surrogate marker protein, as diagrammed herein. The pool of the best individual sequences is recovered from the cells selected by flow cytometry-based sorting. An advantage of this approach is that very large numbers ( $>10^7$ ) can be evaluated in a single vial experiment.

20

### 2.5.3. ADDITIONAL IN VITRO SCREENING METHODS

Screening for improved vaccination properties using various *in vitro* testing methods such as screening for improved adjuvant activity and immunostimulatory properties.

25

Genetic vaccine vectors and vector modules can be screened for improved vaccination properties using various *in vitro* testing methods that are known to those of skill in the art. For example, the optimized genetic vaccines can be tested

for their effect on induction of proliferation of the particular lymphocyte type of interest, e.g., B cells, T cells, T cell lines, and T cell clones. This type of screening for improved adjuvant activity and immunostimulatory properties can be performed using, for example, human or mouse cells.

5

**Screening for improved vaccination properties using various *in vitro* testing methods such as screening for cytokine production (ELISA and/or cytoplasmic cytokine staining and flow cytometry) or for alterations in the capacity of the vectors to direct  $T_H1/T_H2$  differentiation**

10

A library of genetic vaccine vectors, e.g. obtained either from polynucleotide reassembly (optionally in combination with other directed evolution methods described herein), or of vectors harboring genes encoding cytokines, costimulatory molecules etc.) can be screened for cytokine production (e.g., IL-2, IL-4, IL-5, IL-6, IL- 10, IL- 12, IL- 13, IL- 15, IFN- , TNF- ) by B cells, T cells, monocytes/macrophages, total human PBMC, or (diluted) whole blood. Cytokines can be measured by ELISA or and cytoplasmic cytokine staining and flow cytometry (single-cell analysis). Based on the cytokine production profile, one can screen for alterations in the capacity of the vectors to direct  $T_H1/T_H2$  differentiation (as evidenced, for example, by changes in ratios of IL-4/ IFN- , IL-4/IL-2, IL-5/ IFN- , IL-5/IL- 2, IL- 13/ IFN- , IL- 13/IL-2). Induction of APC activation can be detected based on changes in surface expression levels of activation antigens, such as B7-1 (CD80), 137-2 (CD86), MHC class I and II, CD14, CD23, and Fc receptors, and the like.

25

**Analyzing genetic vaccine vectors for their capacity to induce T cell activation through isolating spleen cell of infected mice and studying the capacity of cytotoxic T lymphocytes to lyse infected, autologous target cells**

5           In some embodiments, genetic vaccine vectors are analyzed for their capacity to induce T cell activation. More specifically, spleen cells from injected mice can be isolated and the capacity of cytotoxic T lymphocytes to lyse infected, autologous target cells is studied. The spleen cells are reactivated with the specific antigen *in vitro*. In addition, T helper cell differentiation is analyzed by measuring  
10 proliferation or production of  $T_H1$  (IL-2 and IFN- ) and  $T_H2$  (IL-4 and IL-5) cytokines by ELISA and directly in  $CD4^+$  T cells by cytoplasmic cytokine staining and flow cytometry.

15   **Testing for ability to induce humoral immune responses with assays using, for example, peripheral B lymphocytes from immunized individuals or other assays involving detection of antigen expression by the target cells**

Genetic vaccines and vaccine components can also be tested for ability to  
20 induce humoral immune responses, as evidenced, for example, by induction of B cell production of antibodies specific for an antigen of interest. These assays can be conducted using, for example, peripheral B lymphocytes from immunized individuals. Such assay methods are known to those of skill in the art. Other assays involve detection of antigen expression by the target cells. For example,  
25 FACS selection provides the most efficient method of identifying cells which produce a desired antigen on the cell surface. Another advantage of FACS selection is that one can sort for different levels of expression; sometimes lower expression may be desired. Another method involves panning using monoclonal antibodies on a plate. This method allows large numbers of cells to be handled in

a short time, but the method only selects for highest expression levels. Capture by magnetic beads coated with monoclonal antibodies provides another method of identifying cells which express a particular antigen.

5    **Screening for ability to inhibit proliferation of tumor cell lines *in vitro***

Genetic vaccines and vaccine components that are directed against cancer cells can be screened for their ability to inhibit proliferation of tumor cell lines *in vitro*. Such assays are known in the art. An indication of the efficacy of a genetic vaccine against, for example, cancer or an autoimmune disorder, is the degree of skin inflammation when the vector is injected into the skin of a patient or test animal. Strong inflammation is correlated with strong activation of antigen-specific T cells. Improved activation of tumor-specific T cells may lead to enhanced killing of the tumors. In case of autoantigens, one can add immunomodulators that skew the responses towards T<sub>H</sub>2. Skin biopsies can be taken, enabling detailed studies of the type of immune response that occurs at the sites of each injection (in mice large numbers of injections/vectors can be analyzed) Other suitable screening methods can involve detection of changes in expression of cytokines, chemokines, accessory molecules, and the like, by cells upon challenge by a library of genetic vaccine vectors.

**Expressing the Recombinant Peptides or Polypeptides as Fusions with a Protein Displayed on the Surface of a Replicable Genetic Package**

25

Various screening methods for particular applications are described herein. In several instances, screening involves expressing the recombinant peptides or polypeptides encoded by the experimentally generated polynucleotides of the library as fusions with a protein that is displayed on the surface of a replicable

genetic package. For example, phage display can be used. See, e.g., Cwirla et al.,  
Proc. Natl. Acad. Sci. USA 87: 6378-6382 (1990); Devlin et al., Science 249:  
404-406 (1990), Scott &#0000; Ladner et al., US 5,571,698. Other replicable  
genetic packages include, for example, bacteria, eukaryotic viruses, yeast, and  
5 spores.

**Purification and *in vitro* analysis of recombinant nucleic acids and  
polypeptides**

10

Once stochastic (e.g. polynucleotide shuffling & interrupted synthesis)  
and/or non-stochastic polynucleotide reassembly has been performed, the  
resulting library of experimentally generated polynucleotides can be subjected to  
purification and preliminary analysis *in vitro*, in order to identify the most  
15 promising candidate recombinant nucleic acids. Advantageously, the assays can  
be practiced in a high-throughput format. For example, to purify individual  
experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide  
site-saturation mutagenesis) recombinant antigens, clones can robotically picked  
into 96- well formats, grown, and, if desired, frozen for storage.

20

Whole cell lysates (V-antigen), periplasmic extracts, or culture  
supernatants (toxins) can be assayed directly by ELISA as described below, but  
high throughput purification is sometimes also needed. Affinity chromatography  
using immobilized antibodies or incorporation of a small nonimmunogenic  
25 affinity tag such as a hexahistidine peptide with immobilized metal affinity  
chromatography will allow rapid protein purification. High binding-capacity  
reagents with 96-well filter bottom plates provide a high throughput purification  
process. The scale of culture and purification will depend on protein yield, but  
initial studies will require less than 50 micrograms of protein. Antigens showing

improved properties can be purified in larger scale by FPLC for re-assay and animal challenge studies.

5 In some embodiments, the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigen-encoding polynucleotides are assayed as genetic vaccines. Genetic vaccine vectors containing the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigen sequences can be prepared using robotic colony picking and subsequent robotic plasmid purification. Robotic  
10 plasmid purification protocols are available that allow purification of 600-800 plasmids per day. The quantity and purity of the DNA can also be analyzed in 96-well plates, for example. In a presently preferred embodiment, the amount of DNA in each sample is robotically normalized, which can significantly reduce the variation between different batches of vectors.

15

Once the proteins and/or nucleic acids are picked and purified as desired, they can be subjected to any of a number of *in vitro* analysis methods. Such screenings include, for example, phage display, flow cytometry, and ELISA assays to identify antigens that are efficiently expressed and have multiple  
20 epitopes and a proper folding pattern. In the case of bacterial toxins, the libraries may also be screened for reduced toxicity in mammalian cells.

As one example, to identify recombinant antigens that are cross-reactive, one can use a panel of monoclonal antibodies for screening. A humoral immune  
25 response generally targets multiple regions of antigenic proteins. Accordingly, monoclonal antibodies can be raised against various regions of immunogenic proteins (Alving et al. (1995) Immunol. Rev. 145: 5). In addition, there are several examples of monoclonal antibodies that only recognize one strain of a given pathogen, and by definition, different serotypes of pathogens are recognized by

different sets of antibodies. For example, a panel of monoclonal antibodies have been raised against VEE envelope proteins, thus providing a means to recognize different subtypes of the virus (Roehrig and Bolin (1997) *J Clin. Microbiol.* 35: 1887). Such antibodies, combined with phage display and ELISA screening, can  
5 be used to enrich recombinant antigens that have epitopes from multiple pathogen strains. Flow cytometry based cell sorting will further allow for the selection of variants that are most efficiently expressed.

Phage display provides a powerful method for selecting proteins of  
10 interest from large libraries (Bass et al. (1990) *Proteins: Struct. Funct. Genet.* 8: 309; Lowman and Wells (1991) *Methods: A Companion to Methods Enz.* 3(3);205-216. Lowman and Wells (1993) *J Mol. Biol.* 234;564-578). Some recent reviews on the phage display technique include, for example, McGregor (1996) *Mol Biotechnol.* 6(2):15 5 -62; Dunn (1996) *Curr. Opin. Biotechnol.* 7(5):547-53;  
15 Hill et al. (1996) *Mol Microbiol* 20(4):685-92; Phage Display of Peptides and Proteins: A Laboratory Manual. BK. Kay, J. Winter, J. McCafferty eds., Academic Press 1996; O'Neil et al. (1995) *Curr. Opin. Struct. Biol.* 5(4):443-9; Phizicky et al. (1995) *Microbiol Rev.* 59(1):94-123; Clackson et al. (1994) *Trends Biotechnol.* 12(5):173-84; Felici et al. (1995) *Biotechnol. Annu. Rev.* 1: 149-83;  
20 Burton (1995) *Immunotechnology* 1(2):87-94.) See, also, Cwirla et al., *Proc. Natl. Acad. Sci. USA* 87: 6378-6382 (1990); Devlin et al., *Science* 249: 404-406 (1990), Scott & Smith, *Science* 249: 386-388 (1990); Ladner et al., US 5,571,698. Each phage particle displays a unique variant protein on its surface and packages the gene encoding that particular variant. The experimentally evolved (e.g. by  
25 polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes for the antigens are fused to a protein that is expressed on the phage surface, e.g., gene III of phage M 13, and cloned into phagemid vectors. In a presently preferred embodiment, a suppressible stop codon (e.g., an amber stop codon) separates the genes so that in a suppressing strain of *E. coli*, the antigen-gIIIp



fusion is produced and becomes incorporated into phage particles upon infection with M 13 helper phage. The same vector can direct production of the unfused antigen alone in a nonsuppressing *E. coli* for protein purification.

5

#### **Most Frequently Used Genetic Packages for Display Libraries**

The genetic packages most frequently used for display libraries are bacteriophage, particularly filamentous phage, and especially phage M13, Fd and  
10 F1. Most work has involved inserting libraries encoding polypeptides to be displayed into either gIII or gVIII of these phage forming a fusion protein. See, e.g., Dower, WO 91/19818; Devlin, WO 91/18989; MacCafferty, WO 92/01047 (gene III); Huse, WO 92/06204; Kang, WO 92/18619 (gene VIII). Such a fusion  
15 protein comprises a signal sequence, usually but not necessarily, from the phage coat protein, a polypeptide to be displayed and either the gene III or gene VIII protein or a fragment thereof. Exogenous coding sequences are often inserted at or near the N-terminus of gene III or gene VIII although other insertion sites are possible.

20

#### **Use of Eukaryotic Viruses to Display Polypeptides**

Eukaryotic viruses can be used to display polypeptides in an analogous manner. For example, display of human heregulin fused to gp70 of Moloney  
25 murine leukemia virus has been reported by Han et al., Proc. Natl. Acad. Sci. USA 92: 9747-9751 (1995). Spores can also be used as replicable genetic packages. In this case, polypeptides are displayed from the outer surface of the spore. For example, spores from *B. subtilis* have been reported to be suitable. Sequences of coat proteins of these spores are provided by Donovan et al., J. Mol.

Biol. 196, 1-10 (1987). Cells can also be used as replicable genetic packages. Polypeptides to be displayed are inserted into a gene encoding a cell protein that is expressed on the cells surface. Bacterial cells including *Salmonella typhimurium*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*,  
5 *Klebsiella pneumonia*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Bacteroides nodosus*, *Moraxella bovis*, and especially *Escherichia coli* are preferred. Details of outer surface proteins are discussed by Ladner et al., US 5,571,698 and references cited therein. For example, the *lamB*, protein of *E. coli* is suitable.

10

#### Establishment of a Physical Association Between Polypeptides and Their Genetic Material

15 A basic concept of display methods that use phage or other replicable genetic package is the establishment of a physical association between DNA encoding a polypeptide to be screened and the polypeptide. This physical association is provided by the replicable genetic package, which displays a polypeptide as part of a capsid enclosing the genome of the phage or other  
20 package, wherein the polypeptide is encoded by the genome. The establishment of a physical association between polypeptides and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different polypeptides. Phage displaying a polypeptide with affinity to a target, e.g., a receptor, bind to the target and these phage are enriched by affinity screening to  
25 the target. The identity of polypeptides displayed from these phage can be determined from their respective genomes.

Using these methods a polypeptide identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means, or the

polynucleotide that encodes the peptide or polypeptide can be used as part of a genetic vaccine.

5 Variants with specific binding properties, in this case binding to family-specific antibodies, are easily enriched by panning with immobilized antibodies. Antibodies specific for a single family are used in each round of panning to rapidly select variants that have multiple epitopes from the antigen families. For example, A-family specific antibodies can be used to select those experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation  
10 mutagenesis) clones that display A-specific epitopes in the first round of panning. A second round of panning with B-specific antibodies will select from the "A" clones those that display both A- and B-specific epitopes. A third round of panning with C- specific antibodies will select for variants with A, B, and C epitopes. A continual selection exists during this process for clones that express  
15 well in E. coli and that are stable throughout the selection. Improvements in factors such as transcription, translation, secretion, folding and stability are often observed and will enhance the utility of selected clones for use in vaccine production.

20 Phage ELISA methods can be used to rapidly characterize individual variants. These assays provide a rapid method for quantitation of variants without requiring purification of each protein. Individual clones are arrayed into 96-well plates, grown, and frozen for storage. Cells in duplicate plates are infected with helper phage, grown overnight and pelleted by centrifugation. The supernatants  
25 containing phage displaying particular variants are incubated with immobilized antibodies and bound clones are detected by anti- M13 antibody conjugates. Titration series of phage particles, immobilized antigen, and/or soluble antigen competition binding studies are all highly effective means to quantitate protein

binding. Variant antigens displaying multiple epitopes will be further studied in appropriate animal challenge models.

Several groups have reported an *in vitro* ribosome display system for the  
5 screening and selection of mutant proteins with desired properties from large  
libraries. This technique can be used similarly to phage display to select or enrich  
for variant antigens with improved properties such as broad cross reactivity to  
antibodies and improved folding (see, e.g., Hanes et al. (1997) Proc. Nat'l. Acad.  
Sci. USA 94(10):493 7-42; Mattheakis et al. (1994) Proc. Nat'l. Acad. Sci. USA  
10 91(19):9022-6; He et al. (1997) Nucl. Acids Res. (24):5132-4; Nemoto et al.  
(1997) FEBS Lett. 414(2):405-8).

Other display methods exist to screen antigens for improved properties  
such as increased expression levels, broad cross reactivity, enhanced folding and  
15 stability. These include, but are not limited to display of proteins on intact *E. coli*  
or other cells (e.g., Francisco et al. (1993) Proc. Nat'l. Acad. Sci. USA 90: 1044-  
10448; Lu et al. (1995) *BiolTechnology* 13: 366-372). Fusions of experimentally  
evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation  
mutagenesis) antigens to DNA-binding proteins can link the antigen protein to its  
20 gene in an expression vector (Schatz et al. (1996) *Methods Enzymol.* 267: 171-91;  
Gates et al. (1996) *J Mol. Biol.* 255: 373-86.) The various display methods and  
ELISA assays can be used to screen for experimentally evolved (e.g. by  
polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis)  
antigens with improved properties such as presentation of multiple epitopes,  
25 improved immunogenicity, increased expression levels, increased folding rates  
and efficiency, increased stability to factors such as temperature, buffers, solvents,  
improved purification properties, etc. Selection of experimentally evolved (e.g. by  
polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis)  
antigens with improved expression, folding, stability and purification profile

under a variety of chromatographic conditions can be very important improvements to incorporate for the vaccine manufacturing process.

To identify recombinant antigenic polypeptides that exhibit improved expression in a host cell, flow cytometry is a useful technique.

5

Flow cytometry provides a method to efficiently analyze the functional properties of millions of individual cells. One can analyze the expression levels of several genes simultaneously, and flow cytometry-based cell sorting allows for the selection of cells that display properly expressed antigen variants on the cell surface or in the cytoplasm. Very large numbers ( $> 10^7$ ) of cells can be evaluated in a single vial experiment, and the pool of the best individual sequences can be recovered from the sorted cells. These methods are particularly useful in the case of, for example, Hantaan virus glycoproteins, which are generally very poorly expressed in mammalian cells. This approach provides a general solution to improve expression levels of pathogen antigens in mammalian cells, a phenomenon that is critical for the function of genetic vaccines.

To use flow cytometry to analyze polypeptides that are not expressed on the cell surface, one can engineer the experimentally generated polynucleotides in the library such that the polynucleotide is expressed as a fusion protein that has a region of amino acids which is targeted to the cell membrane. For example, the region can encode a hydrophobic stretch of C-terminal amino acids which signals the attachment of a phosphoinositol- glycan (PIG) terminus on the expressed protein and directs the protein to be expressed on the surface of the transfected cell (Whitehorn et al. (1995) Biotechnology (N Y) 13:1215-9). With an antigen that is naturally a soluble protein, this method will likely not affect the three dimensional folding of the protein in this engineered fusion with a new C-terminus. With an antigen that is naturally a transmembrane protein (e.g., a

surface membrane protein on pathogenic viruses, bacteria, protozoa or tumor cells) there are at least two possibilities.

5 First, the extracellular domain can be engineered to be in fusion with the C-terminal sequence for signaling PIG-linkage. Second, the protein can be expressed *in toto* relying on the signaling of the host cell to direct it efficiently to the cell surface. In a minority of cases, the antigen for expression will have an endogenous PIG terminal linkage (e.g., some antigens of pathogenic protozoa).

10 Those cells expressing the antigen can be identified with a fluorescent monoclonal antibody specific for the C-terminal sequence on PIG-linked forms of the surface antigen. FACS analysis allows quantitative assessment of the level of expression of the correct form of the antigen on the cell population. Cells expressing the maximal level of antigen are sorted and standard molecular  
15 biology methods are used to recover the plasmid DNA vaccine vector that conferred this reactivity. An alternative procedure that allows purification of all those cells expressing the antigen (and that may be useful prior to loading onto a cell sorter since antigen expressing cells may be a very small minority population), is to rosette or pan-purify the cells expressing surface antigen.  
20 Rosettes can be formed between antigen expressing cells and erythrocytes bearing covalently coupled antibody to the relevant antigen. These are readily purified by unit gravity sedimentation. Panning of the cell population over petri dishes bearing immobilized monoclonal antibody specific for the relevant antigen can also be used to remove unwanted cells.

25

In the high throughput assays of the invention, it is possible to screen up to several thousand different experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) variants in a single day. For example, each well of a microtiter plate can be used to run a separate

assay, or, if concentration or incubation time effects are to be observed, every 5 -  
10 wells can test a single variant. Thus, a single standard microtiter plate can  
assay about 100 (e.g., 96) reactions. If 1536 well plates are used, then a single  
plate can easily assay from about 100 to about 1500 different reactions. It is  
5 possible to assay several different plates per day; assay screens for up to about  
6,000-20,000 different assays (i.e., involving different nucleic acids, encoded  
proteins, concentrations, etc.) is possible using the integrated systems of the  
invention. More recently, microfluidic approaches to reagent manipulation have  
been developed, e.g., by Caliper Technologies (Palo Alto, CA).

10

In one aspect, library members, e.g., cells, viral plaques, or the like, are  
separated on solid media to produce individual colonies (or plaques). Using an  
automated colony picker (e.g., the Q-bot, Genetix, U.K.), colonies or plaques are  
identified, picked, and up to 10,000 different mutants inoculated into 96 well  
15 microtiter dishes, optionally containing glass balls in the wells to prevent  
aggregation. The Q-bot does not pick an entire colony but rather inserts a pin  
through the center of the colony and exits with a small sampling of cells (or  
viruses in plaque applications). The time the pin is in the colony, the number of  
dips to inoculate the culture medium, and the time the pin is in that medium each  
20 effect inoculum size, and each can be controlled and optimized. The uniform  
process of the Q-bot decreases human handling error and increases the rate of  
establishing cultures (roughly 10,000/4 hours). These cultures are then shaken in a  
temperature and humidity controlled incubator. The glass balls in the microtiter  
plates act to promote uniform aeration of cells dispersal of cells, or the like,  
25 similar to the blades of a fermentor. Clones from cultures of interest can be cloned  
by limiting dilution. Plaques or cells constituting libraries can also be screened  
directly for production of proteins, either by detecting hybridization, protein  
activity, protein binding to antibodies, or the like.

The ability to detect a subtle increase in the performance of a experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library member over that of a parent strain relies on the sensitivity of the assay. The chance of finding the organisms having an improvement in ability to induce an immune response is increased by the number of individual mutants that can be screened by the assay. To increase the chances of identifying a pool of sufficient size, a prescreen that increases the number of mutants processed by 10-fold can be used. The goal of the prescreen will be to quickly identify mutants having equal or better product titers than the parent strain(s) and to move only these mutants forward to liquid cell culture for subsequent analysis.

A number of well known robotic systems have also been developed for solution phase chemistries useful in assay systems. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a scientist. Any of the above devices are suitable for use with the present invention, e.g., for high- throughput screening of molecules encoded by codon-altered nucleic acids. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein with reference to the integrated system will be apparent to persons skilled in the relevant art.

High throughput screening systems are commercially available (see, e.g., Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the



microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization.

5           The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like. Microfluidic approaches to reagent manipulation have also been developed, e.g., by Caliper Technologies (Palo Alto, CA).

10

Optical images viewed (and, optionally, recorded) by a camera or other recording device (e.g., a photodiode and data storage device) are optionally further processed in any of the embodiments herein, e.g., by digitizing the image and/or storing and analyzing the image on a computer. As noted above, in some applications, the signals resulting from assays are florescent, making optical detection approaches appropriate in these instances. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image, e.g., using PC (Intel x86 or Pentium chip- compatible DOS, OS2 WINDOWS, WINDOWS NT or  
15           VIMOWS95 based machines), MACINTOSH, or LTNIX based (e.g., SLJN work  
20           station) computers.

One conventional system carries light from the assay device to a cooled charge-coupled device (CCD) camera, in common use in the art. A CCD camera  
25           includes an array of picture elements (pixels). The light from the specimen is imaged on the CCD. Particular pixels corresponding to regions of the specimen (e.g., individual hybridization sites on an array of biological polymers) are sampled to obtain light intensity readings for each position. Multiple pixels are processed in parallel to increase speed. The apparatus and methods of the

invention are easily used for viewing any sample, e.g., by fluorescent or dark field microscopic techniques.

Integrated systems for analysis in the present invention typically include a  
5 digital computer with high-throughput liquid control software, image analysis  
software, data interpretation software, a robotic liquid control armature for  
transferring solutions from a source to a destination operably linked to the digital  
computer, an input device (e.g., a computer keyboard) for entering data to the  
digital computer to control high throughput liquid transfer by the robotic liquid  
10 control armature and, optionally, an image scanner for digitizing label signals  
from labeled assay component. The image scanner interfaces with the image  
analysis software to provide a measurement of optical intensity. Typically, the  
intensity measurement is interpreted by the data interpretation software to show  
whether the optimized recombinant antigenic polypeptide products are produced.  
15

#### 2.5.4. ANTIGEN LIBRARY IMMUNIZATION

20 In a presently preferred embodiment, antigen library immunization (ALI)  
is used to identify optimized recombinant antigens that have improved  
immunogenicity. ALI involves introduction of the library of recombinant antigen-  
encoding nucleic acids, or the recombinant antigens encoded by the  
experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide  
25 site-saturation mutagenesis) nucleic acids, into a test animal. The animals are then  
subjected to *in vivo* challenge using live pathogens. Neutralizing antibodies and  
cross-protective immune responses are studied after immunization with the entire  
libraries, pools and/or individual antigen variants.

Methods of immunizing test animals are well known to those of skill in the art. In presently preferred embodiments, test animals are immunized twice or three times at two week intervals. One week after the last immunization, the animals are challenged with live pathogens (or mixtures of pathogens), and the survival and symptoms of the animals is followed. Immunizations using test animal challenge are described in, for example, Roggenkamp et al. (1997) Infect. Immun. 65: 446; Woody et al. (1997) Vaccine 2: 133; Agren et al. (1997) J Immunol. 158: 3936; Konishi et al. (1992) Virology 190: 454; Kinney et al. (1988) J Virol. 62: 4697; Iacono-Connors et al. (1996) Virus Res. 43: 125; Kochel et al. (1997) Vaccine 15: 547; and Chu et al. (1995) J Virol. 69: 6417.

The immunizations can be performed by injecting either the experimentally generated polynucleotides themselves, i.e., as a genetic vaccine, or by immunizing the animals with polypeptides encoded by the experimentally generated polynucleotides. Bacterial antigens are typically screened primarily as recombinant proteins, whereas viral antigens are preferably analyzed using genetic vaccinations.

To dramatically reduce the number of experiments required to identify individual antigens having improved immunogenic properties, one can use pooling and deconvolution, as diagrammed herein. Pools of recombinant nucleic acids, or polypeptides encoded by the recombinant nucleic acids, are used to immunize test animals. Those pools that result in protection against pathogen challenge are then subdivided and subjected to additional analysis. The high throughput *in vitro* approaches described above can be used to identify the best candidate sequences for the *in vivo* studies.

The challenge models that can be used to screen for protective antigens include pathogen and toxin models, such as *Yersinia* bacteria, bacterial toxins

(such as Staphylococcal and Streptococcal enterotoxins, *E. coli*/*V. cholerae* enterotoxins), Venezuelan equine encephalitis virus (VEE), Flaviviruses (Japanese encephalitis virus, Tick-borne encephalitis virus, Dengue virus), Hantaan virus, Herpes simplex, influenza virus (e.g., Influenza A virus), Vesicular Steatitis Virus, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Toxoplasma gondii*, *Plasmodium yoelii*, Herpes simplex, influenza virus (e.g., Influenza A virus), and Vesicular Steatitis Virus. However, the test animals can also be challenged with tumor cells to enable screening of antigens that efficiently protect against malignancies. Individual experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigens or pools of antigens are introduced into the animals intradermally, intramuscularly, intravenously, intratracheally, anally, vaginally, orally, or intraperitoneally and antigens that can prevent the disease are chosen, when desired, for further rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection. Eventually, the most potent antigens, based on *in vivo* data in test animals and comparative *in vitro* studies in animals and man, are chosen for human trials, and their capacity to prevent and treat human diseases is investigated.

In some embodiments, antigen library immunization and pooling of individual clones is used to immunize against a pathogen strain that was not included in the sequences that were used to generate the library. The level of crossprotection provided by different strains of a given pathogen can significantly. However, homologous titer is always higher than heterologous titer. Pooling and deconvolution is especially efficient in models where minimal protection is provided by the wild-type antigens used as starting material for reassembly (optionally in combination with other directed evolution methods described herein). This approach can be taken, for example, when evolving the V-antigen of *Yersinae* or Hantaan virus glycoproteins.

In some embodiments, the desired screening involves analysis of the immune response based on immunological assays known to those skilled in the art. Typically, the test animals are first immunized and blood or tissue samples are collected for example one to two weeks after the last immunization. These studies  
5 enable one to one can measure immune parameters that correlate to protective immunity, such as induction of specific antibodies (particularly IgG) and induction of specific T lymphocyte responses, in addition to determining whether an antigen or pools of antigens provides protective immunity.

10 Spleen cells or peripheral blood mononuclear cells can be isolated from immunized test animals and measured for the presence of antigen-specific T cells and induction of cytokine synthesis. ELISA, ELISPOT and cytoplasmic cytokine staining, combined with flow cytometry, can provide such information on a single-cell level.

15 Common immunological tests that can be used to identify the efficacy of immunization include antibody measurements, neutralization assays and analysis of activation levels or frequencies of antigen presenting cells or lymphocytes that are specific for the antigen or pathogen. The test animals that can be used in such  
20 studies include, but are not limited to, mice, rats, guinea pigs, hamsters, rabbits, cats, dogs, pigs and monkeys.

Monkey is a particularly useful test animal because the MHC molecules of monkeys and humans are very similar. Virus neutralization assays are useful for  
25 detection of antibodies that not only specifically bind to the pathogen, but also neutralize the function of the virus. These assays are typically based on detection of antibodies in the sera of immunized animal and analysis of these antibodies for their capacity to inhibit viral growth in tissue culture cells. Such assays are known to those skilled in the art. One example of a virus neutralization assay is described

by Dolin R (J. Infect. Dis. 1995, 172:1175-83). Virus neutralization assays provide means to screen for antigens that also provide protective immunity.

In some embodiments, experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigens are screened for their capacity to induce T cell activation *in vivo*. More specifically, peripheral blood mononuclear cells or spleen cells from injected mice can be isolated and the capacity of cytotoxic T lymphocytes to lyse infected, autologous target cells is studied. The spleen cells can be reactivated with the specific antigen *in vitro*. In addition, T helper cell activation and differentiation is analyzed by measuring cell proliferation or production of T<sub>H</sub> (IL-2 and IFN- $\gamma$ ) and T<sub>H</sub>2 (IL-4 and IL-5) cytokines by ELISA and directly in CD4<sup>+</sup> T cells by cytoplasmic cytokine staining and flow cytometry. Based on the cytokine production profile, one can also screen for alterations in the capacity of the antigens to direct T<sub>H</sub>1/T<sub>H</sub>2 differentiation (as evidenced, for example, by changes in ratios of IL-4/IFN- $\gamma$ , IL-4/IL-2, IL-5/IFN- $\gamma$ , IL-5/IL-2, IL-13/IFN- $\gamma$ , IL-13/IL-2). The analysis of the T cell activation induced by the antigen variants is a very useful screening method, because potent activation of specific T cells *in vivo* correlates to induction of protective immunity.

20

The frequency of antigen-specific CD8<sup>+</sup> T cells *in vivo* can also be directly analyzed using tetramers of MHC class I molecules expressing specific peptides derived from the corresponding pathogen antigens (Ogg and McMichael, Curr. Opin. Immunol. 1998, 10:393-6; Altman et al., Science 1996, 274:94-6).

The binding of the tetramers can be detected using flow cytometry, and will provide information about the efficacy of the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigens to induce activation of specific T cells. For example, flow cytometry and tetramer stainings provide an efficient method of identifying T cells that are

specific to a given antigen or peptide. Another method involves panning using plates coated with tetramers with the specific peptides. This method allows large numbers of cells to be handled in a short time, but the method only selects for highest expression levels. The higher the frequency of antigen-specific T cells *in vivo* is, the more efficient the immunization has been, enabling identification of the antigen variants that have the most potent capacity to induce protective immune responses. These studies are particularly useful when conducted in monkeys, or other primates, because the MHC class I molecules of humans mimic those of other primates more closely than those of mice.

10

Measurement of the activation of antigen presenting cells (APC) in response to immunization by antigen variants is another useful screening method. Induction of APC activation can be detected based on changes in surface expression levels of activation antigens, such as 137-1 (CD80), 137-2 (CD86), MHC class I and II, CD14, CD23, and Fc receptors, and the like.

15

Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) cancer antigens that induce cytotoxic T cells that have the capacity to kill cancer cells can be identified by measuring the capacity of T cells derived from immunized animals to kill cancer cells *in vitro*. Typically the cancer cells are first labeled with radioactive isotopes and the release of radioactivity is an indication of tumor cell killing after incubation in the presence of T cells from immunized animals. Such cytotoxicity assays are known in the art.

20

25

An indication of the efficacy of an antigen to activate T cells specific for, for example, cancer antigens, allergens or autoantigens, is also the degree of skin inflammation when the antigen is injected into the skin of a patient or test animal. Strong inflammation is correlated with strong activation of antigen-specific T

- cells. Improved activation of tumor- specific T cells may lead to enhanced killing of the tumors. In case of autoantigens, one can add immunomodulators that skew the responses towards  $T_H2$ , whereas in the case of allergens a  $T_H1$  response is desired. Skin biopsies can be taken, enabling detailed studies of the type of
- 5 immune response that occurs at the sites of each injection (in mice and monkeys large numbers of injections/antigens can be analyzed). Such studies include detection of changes in expression of cytokines, chemokines, accessory molecules, and the like, by cells upon injection of the antigen into the skin.
- 10 To screen for antigens that have optimal capacity to activate antigen-specific T cells, peripheral blood mononuclear cells from previously infected or immunized humans individuals can be used. This is a particularly useful method, because the MHC molecules that will present the antigenic peptides are human MHC molecules. Peripheral blood mononuclear cells or purified professional
- 15 antigen-presenting cells (APCs) can be isolated from previously vaccinated or infected individuals or from patients with acute infection with the pathogen of interest. Because these individuals have increased frequencies of pathogen-specific T cells in circulation, antigens expressed in PBMCs or purified APCs of these individuals will induce proliferation and cytokine production by antigen-
- 20 specific  $CD4^+$  and  $CD8^+$  T cells. Thus, antigens that simultaneously harbor epitopes from several antigens can be recognized by their capacity to stimulate T cells from various patients infected or immunized with different pathogen antigens, cancer antigens, autoantigens or allergens. One buffy coat derived from a blood donor contains lymphocytes from 0.5 liters of blood, and up to  $10^4$  PBMC
- 25 can be obtained, enabling very large screening experiments using T cells from one donor.

When healthy vaccinated individuals (lab volunteers) are studied, one can make EBV-transformed B cell lines from these individuals. These cell lines can



be used as antigen presenting cells in subsequent experiments using blood from the same donor; this reduces interassay and donor-to-donor variation. In addition, one can make antigen-specific T cell clones, after which antigen variants are introduced to EBV transformed B cells. The efficiency with which the  
5 transformed B cells induce proliferation of the specific T cell clones is then studied. When working with specific T cell clones, the proliferation and cytokine synthesis responses are significantly higher than when using total PBMCs, because the frequency of antigen-specific T cells among PBMC is very low.

10 CTL epitopes can be presented by most cells types since the class I major histocompatibility complex (MHC) surface glycoproteins are widely expressed. Therefore, transfection of cells in culture by libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation  
15 class I epitope presentation. If specific CTLs directed to a given epitope have been isolated from an individual, then the co-culture of the transfected presenting cells and the CTLs can lead to release by the CTLs of cytokines, such as IL-2, IFN- , or TNF, if the epitope is presented. Higher amounts of released TNF will correspond to more efficient processing and presentation of the class I epitope  
20 from the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis), evolved sequence. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) antigens that induce cytotoxic T cells that have the capacity to kill infected cells can also be identified by measuring the capacity of T cells derived  
25 from immunized animals to kill infected cells *in vitro*. Typically the target cells are first labeled with radioactive isotopes and the release of radioactivity is an indication of target cell killing after incubation in the presence of T cells from immunized animals. Such cytotoxicity assays are known in the art.

A second method for identifying optimized CTL epitopes does not require the isolation of CTLs reacting with the epitope. In this approach, cells expressing class I MHC surface glycoproteins are transfected with the library of evolved sequences as above. After suitable incubation to allow for processing and presentation, a detergent soluble extract is prepared from each cell culture and after a partial purification of the MHC-epitope complex (perhaps optional) the products are submitted to mass spectrometry (Henderson et al. (1993) Proc. Nat'l. Acad. Sci. USA 90: 10275-10279). Since the sequence is known of the epitope whose presentation to be increased, one can calibrate the mass spectrogram to identify this peptide. In addition, a cellular protein can be used for internal calibration to obtain a quantitative result; the cellular protein used for internal calibration could be the MHC molecule itself. Thus one can measure the amount of peptide epitope bound as a proportion of the MHC molecules.

15

#### 2.5.5. SCREENING FOR OPTIMAL INDUCTION OF PROTECTIVE IMMUNITY

Vectors that can provide efficient, protective immunity are selected using lethal infection models to choose vectors that can prevent the disease for further rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection

To select genetic vaccine vectors that provide efficient protective immunity, one can screen the vector libraries in a test mammal using lethal infection models, such as *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Toxoplasma gondii*, *Plasmodium yoelii*, Herpes simplex, influenza virus (e.g., Influenza A virus), and Vesicular Stomatitis Virus. Pools of genetic vaccine vectors or individual vectors are introduced into

the animals intradermally, intramuscularly, intravenously, intratracheally, anally, vaginally, orally, or intraperitoneally and vectors that can prevent the disease are chosen for further rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

5

**Examples: anti-IL-4 mAbs or recombinant IL-12; recombinant IL-12**  
**(advantage of latter model is that infection occurs through lung, common**  
**route of human pathogen invasion)**

10

As an example, optimal vectors can be screened in mice infected with *Leishmania major* parasites. When injected into footpads of BALB/c mice, these parasites cause a progressive infection later resulting in a disseminated disease with fatal outcome, which can be prevented by anti-IL-4 mAbs or recombinant IL-12 (Chatelain et al. (1992) J. Immunol. 148: 1182-1187). Pools of plasmids can be injected intravenously, intraperitoneally or into footpads of these mice, and pools that can prevent the disease are chosen for further analysis and screened for vectors that can cure existing infections. The size of the footpad swelling can be followed visually providing simple yet precise monitoring of the disease progression. Mice can be infected intratracheally with *Klebsiella pneumoniae* resulting in lethal pneumonia, which can be prevented by recombinant IL-12 (Greenberger et al. (1996) J Immunol. 157: 3006-3012). The advantage of this model is that the infection occurs through the lung, which is a common route of human pathogen invasion. The vectors can be given to the lung together with the pathogen or they can be administered after symptoms are evident in order to screen for vectors that can cure established infections.

20

25

**Example: Influenza- provides a way to screen for vectors that provide protection at very low quantities of DNA and/or high virus concentrations, and it also allows one to analyze the levels of antigen specific Abs and CTLs induced *in vivo***

5

In another example, the genetic vaccines are a mouse vaccination model for Influenza A virus. Influenza was one of the first models in which the efficacy of genetic vaccines was demonstrated (Ulmer et al. (1993) Science 259: 1745-1749). Several Influenza strains are lethal in mice providing an easy means to  
10 screen for efficacy of genetic vaccines.

For example, Influenza virus strain A/PR/8/34, which is available through the American Type Culture Collection (ATCC VR-95), causes lethal infection, but 100% survival can be obtained when the mice are immunized with and influenza  
15 hemagglutinin (HA) genetic vaccine (Deck et al. (1997) Vaccine 15: 71-78). This model provides a way to screen for vectors that provide protection at very low quantities of DNA and/or high virus concentrations, and it also allows one to analyze the levels of antigen specific Abs and CTLs induced *in vivo*.

20

**Example: Mycobacterium tuberculosis (partial protection, requires major improvements)**

The genetic vaccine vectors can also be analyzed for their capacity to  
25 provide protection against infections by Mycobacterium tuberculosis. This is an example of a situation where genetic vaccines have provided partial protection, and where major improvements are required.

**Identification of candidate vectors followed by more testing**

Once a number of candidate vectors has been identified, these vectors can be subjected to more detailed analysis in additional models. Testing in other  
5 infectious disease models (such as HSV, Mycoplasma pulmonis, RSV and/or rotavirus) will allow identification of vectors that are optimal in each infectious disease.

**Optimal plasmids from the first round of screening are used as the starting  
10 material for the next round, the successful vectors are sequenced and the corresponding human genes are cloned into genetic vaccine vectors which are characterized *in vitro* for their capacity to induce differentiation of a desired trait.**

15 In each case, the optimal plasmids from the first round of screening can be used as the starting material for the next round of reassembly (optionally in combination with other directed evolution methods described herein), assembly and selection. Vectors that are successful in animal models are sequenced and the corresponding human genes are cloned into genetic vaccine vectors. These  
20 vectors are then characterized *in vitro* for their capacity to induce differentiation of T<sub>H</sub>1/ T<sub>H</sub>2 cells, activation of T<sub>H</sub> cells, cytotoxic T lymphocytes and monocytes/macrophages, or other desired trait. Eventually, the most potent vectors, based on *in vivo* data in mice and comparative *in vitro* studies in mice and man, are chosen for human trials, and their capacity to counteract various human  
25 infectious diseases is investigated.

**Methods for measuring immune parameters that correlate to protective immunity**

- 5           In addition to determining whether a vector pool provides protective immunity, one can measure immune parameters that correlate to protective immunity, such as induction of specific antibodies (particularly IgG) and induction of specific CTL responses. Spleen cells can be isolated from vaccinated mice and measured for the presence of antigen- specific T cells and induction of
- 10    $T_H1$  cytokine synthesis profiles. ELISA and cytoplasmic cytokine staining, combined with flow cytometry, can provide such information on a single-cell level.

15           **2.5.6. SCREENING OF GENETIC VACCINE VECTORS THAT  
ACTIVATE HUMAN ANTIGEN-SPECIFIC LYMPHOCYTE  
RESPONSES**

**Isolation of PBMCs or APCs to screen for vectors with optimal**

20   **immunostimulatory properties for the human immune system**

- To screen for vectors with optimal immunostimulatory properties for the human immune system, peripheral blood mononuclear cells (PBMCs) or purified professional antigen-presenting cells (APCs) can be isolated from previously
- 25   vaccinated or infected individuals or from patients with acute infection with the pathogen of interest.

Genetic vaccine vectors encoding the antigen for which the individuals have specific T cells can be transfected into PBMC and induction of T cell proliferation and cytokine synthesis can be measured; also possible to screen for spontaneous entry of genetic vaccine vector into APCs

5

Because these individuals have increased frequencies of pathogen-specific T cells in circulation, antigens expressed in PBMCs or purified APCs of these individuals will induce proliferation and cytokine production by antigen-specific CD4+ and CD8+ T cells. Thus, genetic vaccine vectors encoding the antigen for which the individuals have specific T cells can be transfected into PBMC of the individuals, after which induction of T cell proliferation and cytokine synthesis can be measured. Alternatively, one can screen for spontaneous entry of the genetic vaccine vector into A-PCs, thus providing a means by which to screen simultaneously for improved transfection efficiency, improved expression of antigen and improved induction of activation of specific T cells. Vectors with the most potent immunostimulatory properties can be screened based on their capacity to induce B cell proliferation and immunoglobulin synthesis. One buffy coat derived from a blood donor contains PBMC lymphocytes from 0.5 liters of blood, and up to  $10^4$  PBMC can be obtained, enabling very large screening experiments using T cells from one donor.

10  
15  
20

Making EBV-transformed B cell lines from healthy vaccinated individuals for subsequent experiments

25

When healthy vaccinated individuals (lab volunteers) are studied, one can make EBV-transformed B cell lines from these individuals. These cell lines can be used as antigen presenting cells in subsequent experiments using blood from the same donor; this reduces interassay and donor-to-donor variation). In addition,

one can make antigen-specific T cell clones, after which genetic vaccines are transfected into EBV transformed B cells.

5    **Efficiency with which the transformed B cells induce proliferation of the specific T cell clones**

          The efficiency with which the transformed B cells induce proliferation of the specific T cell clones is then studied. When working with specific T cell  
10    clones, the proliferation and cytokine synthesis responses are significantly higher than when using total PBMCs, because the frequency of antigen-specific T cells among PBMC is very low.

15    **Transfection of cells in culture by libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) DNA sequences in appropriate expression vectors can lead to class I epitope presentation**

20           CTL epitopes can be presented by most cells types since the class I major histocompatibility complex (MHC) surface glycoproteins are widely expressed. Therefore, transfection of cells in culture by libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation  
25    mutagenesis) DNA sequences in appropriate expression vectors can lead to class I epitope presentation. If specific CTLs directed to a given epitope have been isolated from an individual, then the co-culture of the transfected presenting cells and the CTLs can lead to release by the CTLs of cytokines, such as IL-2, IFN- , or TNF , if the epitope is presented. Higher amounts of released TNF . will correspond to more efficient processing and presentation of the class I epitope



from the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis), evolved sequence.

- 5    **Transfecting cells expressing class I MHC surface glycoproteins with library of evolved sequences, preparing a detergent soluble extract, performing a partial purification of the MHC-epitope complex, and then submitting the products to mass spectrometry**

10            A second method for identifying optimized CTL epitopes does not require the isolation of CTLs reacting with the epitope. In this approach, cells expressing class I MHC surface glycoproteins are transfected with the library of evolved sequences as above. After suitable incubation to allow for processing and presentation, a detergent soluble extract is prepared from each cell culture and  
15    after a partial purification of the MHC-epitope complex (perhaps optional) the products are submitted to mass spectrometry (Henderson et al. (1993) Proc. Nat'l. Acad. Sci. USA 90: 10275-10279). Since the sequence is known of the epitope whose presentation to be increased, one can calibrate the mass spectrogram to identify this peptide. In addition, a cellular protein can be used for internal  
20    calibration to obtain a quantitative result; the cellular protein used for internal calibration could be the MHC molecule itself. Thus one can measure the amount of peptide epitope bound as a proportion of the MHC molecules.

25            **2.5.7. SCID-HUMAN SKIN MODEL FOR VACCINATION STUDIES**

- Use of mouse models in vaccine studies limited in that the MHC molecules in mice and man are substantially different, meaning that proteins and peptides that efficiently induce protective immune responses in mice do not necessarily function in humans

Successful genetic vaccinations require transfection of the target cells after injection of the vector, expression of the desired antigen, processing the antigen in antigen presenting cells, presentation of the antigenic peptides in the context of MHC molecules, recognition of the peptide/MHC complex by T cell receptors, interactions of T cells with B cells and professional APCs and induction of specific T cell and B cell responses. All these events could be differentially regulated in mouse and man. A limitation of mouse models in vaccine studies is the fact that the MHC molecules of mice and man are substantially different. Therefore, proteins and peptides that effectively induce protective immune responses in mice do not necessarily function in humans.

- Mouse models can be used to study human tissues in mice *in vivo* for studies of transfection efficiency, transfer sequences, and gene expression levels

To overcome these limitations mouse models can be used to study human tissues in mice *in vivo*. Live pieces of human skin are xenotransplant onto the back of immunodeficient mice, such as SCID mice, allowing screening of the vector libraries for optimal properties in human cells *in vivo*. Recursive selection of episomal vectors provides strong selection pressure for vectors that remain episomal, yet provide high level of gene expression. These mice provide an excellent model for studies on transfection efficiency, transfer sequences and gene expression levels. In addition, antigen presenting cells (APCs) derived from these

mice can also be used to assess the level of antigens delivered to professional APCs, and to study the capacity of these cells to present antigens and induce activation of antigen-specific CD4+ and CD8+ T cells *in vitro*. Significantly, although SCID mice have severely deficient T and B cell components, antigen  
5 presenting cells (dendritic cells and monocytes) are relatively normal in these mice.

**Rendering immunocompetent mice immunodeficient in order to aid  
10 transplantation of human tissue, enabling vaccine studies in human skin  
xenotransplanted into mice with genetically normal immune systems as well,  
due to the transient nature of the *in vivo* immunosuppression**

In one embodiment of this model system, immunocompetent mice are rendered  
15 immunodeficient in order to enable transplantation of human tissue. For example, blocking of CD28 and CD40 pathways promotes long-term survival of allogeneic skin grafts in mice (Larsen et al. (1996) Nature 381: 434). Because the *in vivo* immunosuppression is transient, this model also enables vaccine studies in human skin xenotransplanted into mice with genetically normal immune systems. Several  
20 methods of blocking CD28- 137 interactions and CD40-CD40 ligand interactions are known to those of skill in the art, including, for example, administration of neutralizing anti-B7-1 and 137- 2 antibodies, soluble CTLA-4, a soluble form of the extracellular portion of CTLA-4, a fusion protein that includes CTLA-4 and an Fc portion of an IgG molecule, and neutralizing anti-CD40 or anti- CD40  
25 ligand antibodies. Additional methods by which one can improve transient immunosuppression include administration of one or more of the following reagents: cyclosporin A, anti-IL-2 receptor - chain Ab, soluble IL-2 receptor, IL-10, and combinations thereof.

A model in which SCID-mice transplanted with human skin are injected with HLA-matched PBMC can be used to analyze vectors that provide long lasting expression *in vivo*. In this model, the vectors are injected, or topically applied, into the human skin.

5

If the HLA-matched PBMC injected into mice contains lymphocytes specific for the vector the transfected cells will be recognized, and eventually destroyed, by these vector-specific lymphocytes, providing the possibility to screen for vectors that efficiently escape destruction

10

Thereafter, HLA-matched PBMC are injected into these mice. If the PBMC contains lymphocytes specific for the vector, the transfected cells will be recognized, and eventually destroyed, by these vector-specific lymphocytes.

15 Therefore, this model provides possibilities to screen for vectors that efficiently escape destruction by the immune cells. It has been shown that human PBLs injected into mice with human skin transplants reject the organ, indicating that the CTLs reach the skin in this model. Obtaining HLA-matching skin and blood is possible (e.g. blood sample and skin graft from a patient undergoing skin removal due to malignancy, or blood and foreskin from the same infant).

20

SCIDhu mouse model: additionally, transplanting human skin allows studies on the efficacy of genetic vaccine vectors following injection to the skin

25

An additional model that is suitable for screening as described herein is the modified SCIDhu mouse model, in which pieces of human fetal thymus, liver and bone marrow are transplanted into SCID mice providing functional human immune system in mice (Roncarolo et al. (1996) Semin. Immunol. 8: 207).

Functional human B and T cells, and APCs can be observed in these mice. When additionally human skin is transplanted, it is likely to allow studies on the efficacy of genetic vaccine vectors following injection into the skin. Cotransplantation of skin is likely to improve the model because it will provide an additional source of professional APCs.

2.5.8. MOUSE MODEL FOR STUDYING THE EFFICIENCY  
OF GENETIC VACCINES IN TRANSFECTING HUMAN  
MUSCLE CELLS AND INDUCING HUMAN IMMUNE  
RESPONSES *IN VIVO*

There is a lack of suitable *in vivo* models for studies of the efficiency of genetic vaccines and the vast majority of studies are performed on the mouse model, in which it is sometimes difficult to predict whether the results obtained reliably predict similar vaccinations in humans because of the complexity of events occurring after genetic vaccination

A lack of suitable *in vivo* models has hampered studies of the efficiency of genetic vaccines in inducing antigen expression in human muscle cells and in inducing specific human immune responses. The vast majority of studies on the capacity of genetic vaccines to transfect muscle cells and to induce specific immune responses *in vivo* have employed a mouse model. Because of the complexity of events occurring after genetic vaccination, however, it is sometimes difficult to predict whether results obtained in the mouse model reliably predict the outcome of similar vaccinations in humans. The events required in successful genetic vaccination include transfection of the cells after delivery of the plasmid, expression of the desired antigen, processing the antigen in antigen presenting cells, presentation of the antigenic peptides in the context of MHC molecules,

recognition of the peptide/MHC complex by T cell receptors, interactions of T cells with B cells and professional antigen presenting cells and finally induction of specific T cell and B cell responses. All these events are likely to be somewhat differentially regulated in mouse and man.

5

**The invention provides an *in vivo* model for human muscle cell transfection**

Muscle tissue, obtained for example from cadavers, is transplanted subcutaneously into immunodeficient mice, which can be transplanted with tissues from other species without rejection. This model system is especially valuable because there is no *in vitro* culture system available for normal muscle cells. Muscle tissue, obtained for example from cadavers, is transplanted subcutaneously into immunodeficient mice. Immunodeficient mice can be transplanted with tissues from other species without rejection. Mice suitable for xenotransplantations include, but are not limited to, SCID mice, nude mice and mice rendered deficient in their genes encoding RAG1 or RAG2 genes. SCID mice and RAG deficient mice lack functional T and B cells, and therefore are severely immunocompromised and are unable to reject transplanted organs. Previous studies indicate that these mice can be transplanted with human tissues, such as skin, spleen, liver, thymus or bone, without rejection (Roncarolo et al. (1996) Semin. Immunol. 8: 207). After transplantation of human fetal lymphoid tissues into SCID mice, functional human immune system can be demonstrated in these mice, a model generally referred to as SCID-hu mice. When human muscle tissue is transplanted into SCID-hu mice, one can not only study transfection efficiency and expression of the desired antigen, but one can also study induction of specific human immune responses induced by genetic vaccines *in vivo*. In this case, muscle and lymphoid organs from the same donor are used. Fetal muscle

also has an advantage in that it contains few mature lymphocytes of donor origin decreasing likelihood of graft versus host reaction.

5    **Genetic vaccine vectors are introduced into the human muscle tissue to study the expression of the antigen of interest**

Once the human muscle tissue is established in the mouse, genetic vaccine vectors are introduced into the human muscle tissue to study the expression of the  
10    antigen of interest. When studying transfection efficiency only, RAG deficient mice are preferred, because these mice never have mature B or T cells in the circulation, whereas "leakiness" of SCID phenotype has been demonstrated which may cause variation in the transplantation efficiency.

15

**Model provides an efficient means to study gene expression in human muscle cells *in vivo*, despite the limited survival of the tissue in mice**

The survival of human muscle tissue in mice is likely to be limited even in  
20    immuno-compromised mice. However, because expression studies can be performed within one or two days, this model provides an efficient means to study gene expression in human muscle cells *in vivo*. A modified SCID-hu mouse model with human muscle transplanted into these mice can be used to study human immune responses in mice *in vivo*.

25

**2.5.9. SCREENING FOR IMPROVED DELIVERY OF VACCINES**

**Identifying genetic vaccine vectors that are capable of being administered in a particular manner**

For certain applications, it is desirable to identify genetic vaccine vectors  
5 that are capable of being administered in a particular manner, for example, orally  
or through the skin. The following screening methods provide suitable assays;  
additional assays are also described herein in conjunction with particular genetic  
vaccine properties for which the assays are especially suitable.

10

**Screening for oral delivery either *in vitro* (based on Caco-2 cells) or *in vivo***

Screening for oral delivery can be performed either *in vitro* or *in vivo*. An  
example of an *in vitro* method is based on Caco-2 (human colon adenocarcinoma)  
15 cells which are grown in tissue culture. When grown on semipermeable filters,  
these cells spontaneously differentiate into cells that resemble human small  
intestine epithelium, both structurally and functionally. Genetic vaccine libraries  
and/or vectors can be placed on one side of the Caco-2 cell layer, and vectors that  
are able to move through the cell layer are detected on the opposite side of the  
20 layer.

Libraries can also be screened for amenability to oral delivery *in vivo*. For  
example, a library of vectors can be administered orally, after which target tissues  
are assayed for presence of vectors. Intestinal epithelium, liver, and the  
25 bloodstream are examples of tissues that can be tested for presence of library  
members. Vectors that are successful in reaching the target tissue can be recovered  
and, if further improvement is desired, used in succeeding rounds of reassembly  
(optionally in combination with other directed evolution methods described  
herein) and selection.



**Apparatus which permits large numbers of vectors to be screened efficiently and can be used to study the effect of large numbers of agents *in vivo***

5

For screening a library of genetic vaccine vectors for ability to transfect cells upon injection into skin or muscle, the invention provides an apparatus which permits large numbers of vectors to be screened efficiently. This apparatus is based on 96-well format and is designed to transfer small volumes (2-5  $\mu$ l) from a microtiter plate to skin or muscle of laboratory animals, such as mice and rats. Moreover, human muscle or skin transplanted into immunodeficient mice can be injected.

The apparatus is designed in such a way that the tips move to fit a microtiter plate. After the reagent of interest has been obtained from the plate, the distance of the tips from each other is decreased to 2-3 mm, enabling transfer of 96 reagents to an area of 1.6 cm x 2.4 cm to 2.4 cm x 3.6 cm. The volume of each sample transferred is electronically controlled. Each reagent is mixed with a marker agent or dye to enable recognition of injection site in the tissue. For example, gold particles of different sizes and shapes are mixed with the reagent of interest, and microscopy and immunohistochemistry can be used to identify each injection site and to study the reaction induced by each reagent. When muscle tissue is injected the injection site is first revealed by surgery.

This apparatus can be used to study the effects of large numbers of agents *in vivo*. For example, this apparatus can be used to screen efficiency of large numbers of different DNA vaccine vectors to transfect human skin or muscle cells transplanted into immunodeficient mice.

#### 2.5.10. ENHANCED ENTRY OF GENETIC VACCINE VECTORS INTO CELLS

- 5    Using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to efficiently improve the capacity of DNA to enter the cytoplasm and subsequently the nucleus of human cells

10        The methods involve subjecting to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly polynucleotides which are involved in cell entry. Such polynucleotides are referred to herein as "transfer sequences" or "transfer modules." Transfer modules can be obtained which increase transfer in a cell- specific manner, or which act in a more general manner. Because the exact sequences that affect DNA binding and

15    transfer are not often known, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly may be the only efficient method to improve the capacity of DNA to enter the cytoplasm and subsequently the nucleus of human cells.

- 20        The stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention provide means for optimizing DNA sequences and the three-dimensional structure of the plasmids for ability to confer upon a vector the ability to enter a cell even
- 25    in the absence of detailed information as to the mechanism by which this effect is achieved

      The methods involve reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a

nucleic acid that comprises a transfer sequence. The first and second forms differ from each other in two or more nucleotides. Suitable substrates include, for example, transcription factor binding sites, CpG sequences, poly A, C, G, T oligonucleotides, non-stochastically generated nucleic acid building blocks, and  
5 random DNA fragments such as, for example, genomic DNA, from human or other mammalian species. It has been suggested that cell surface proteins, such as the macrophage scavenger receptor, may act as receptors for specific DNA binding (Pisetsky (1996) Immunity 5: 303). It is not known whether these  
10 receptors recognize specific DNA sequences or whether they bind DNA in a sequence non-specific manner. However, GGGG tetrads have been shown to enhance DNA binding to cell surfaces (Id.). In addition to the DNA sequence, the three-dimensional structure of the plasmids may play a role in the capacity of these plasmids to enter cells. The stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of  
15 the invention provide means for optimizing such sequences for ability to confer upon a vector the ability to enter a cell even in the absence of detailed information as to the mechanism by which this effect is achieved.

20 Clonal isolates of vectors bearing recombinant segments are used to infect separate cultures of cells and the percentage of vectors which enter cells is then determined by, for example, counting cells expressing a marker expressed by the vectors in the course of transfection

25 The resulting library of recombinant transfer modules are screened to identify at least one optimized recombinant transfer module that enhances the capability of a vector comprising the transfer module to enter a cell of interest. For example, vectors that include a recombinant transfer module can be contacted with a population of cells under conditions conducive to entry of the vector into

the cells, after which the percentage of cells in the population which contain the nucleic acid vector is determined. Preferably, the vector will contain a selectable or screenable marker to facilitate identification of cells which contain the vector. In a preferred embodiment, clonal isolates of vectors bearing recombinant  
5 segments are used to infect separate cultures of cells. The percentage of vectors which enter cells can then be determined by, for example, counting cells expressing a marker expressed by the vectors in the course of transfection.

10 **The reassembly (&/or one or more additional directed evolution methods described herein) and rescreening process can be repeated as necessary, until a transfer module that has sufficient ability to enhance transfer is obtained**

Typically, the reassembly (&/or one or more additional directed evolution  
15 methods described herein) process is repeated by reassembling (&/or subjecting to one or more directed evolution methods described herein) at least one optimized transfer sequence with a further form of the transfer sequence to produce a further library of recombinant transfer modules. The further form can be the same or different from the first and second forms. The new library is screened to identify  
20 at least one further optimized recombinant vector module that exhibits an enhancement of the ability of a genetic vaccine vector that includes the optimized transfer module to enter a cell of interest.

The reassembly (&/or one or more additional directed evolution methods  
25 described herein) and rescreening process can be repeated as necessary, until a transfer module that has sufficient ability to enhance transfer is obtained. After one or more of reassembly (&/or one or more additional directed evolution methods described herein) and screening, vector modules are obtained which are capable of conferring upon a nucleic acid vector the ability to enter at least about

50 percent more target cells than a control vector which does not contain the optimized module, more preferably at least about 75 percent more, and most preferably at least about 95 or 99 percent more target cells than a control vector.

5

For integration by homologous recombination, important factors are the degree and length of homology to chromosomal sequences, the frequency of such sequences in the genome, and the specific sequence mediating homologous recombination; for nonhomologous, illegitimate and site-specific recombination, recombination is mediated by specific sites on the therapy vector which interact with cell encoded recombination proteins

Although for vaccine purposes non-integrating vectors are generally preferred, for some applications it may be desirable to use an integrating vector; for these applications DNA sequences that directly or indirectly affect the efficiency of integration can be included in the genetic vaccine vector. For integration by homologous recombination, important factors are the degree and length of homology to chromosomal sequences, as well as the frequency of such sequences in the genome (*e.g.*, *Alu* repeats). The specific sequence mediating homologous recombination is also important, since integration occurs much more easily in transcriptionally active DNA. Methods and materials for constructing homologous targeting constructs are described by *e.g.*, Mansour (1988) Nature 336:348; Bradley (1992) Bio/Technology 10:534. For nonhomologous, illegitimate and site-specific recombination, recombination is mediated by specific sites on the therapy vector which interact with cell encoded recombination proteins, *e.g.*, Cre/Lox and Flp/Frt systems. See, *e.g.*, Baubonis (1993) Nucleic Acids Res. 21:2025-2029, which reports that a vector including a LoxP site becomes integrated at a LoxP site in chromosomal DNA in the presence of Cre recombinase enzyme.

## 2.6. OPTIMIZATION OF GENETIC VACCINE COMPONENTS

5

### Optimizing properties that can influence the efficacy of a genetic vaccine in modulating an immune response in a mammalian system

Many factors can influence the efficacy of a genetic vaccine in modulating an immune response. The ability of the vector to enter a cell, for example, has a significant effect on the ability of the vector to modulate an immune response. The strength of an immune response is also mediated by the immunogenicity of an antigen expressed by a genetic vaccine vector and the level at which the antigen is expressed. The presence or absence of costimulatory molecules produced by the genetic vaccine vector can affect not only the strength, but also the type of immune response that arises due to introduction of the vector into a mammal. An increase in the persistence of a vector in an organism can lengthen the time of immunomodulation, and also makes feasible self-boosting vectors which do not require multiple administrations to achieve long-lasting protection. The present invention provides methods for optimizing many of these properties, thus resulting in genetic vaccine vectors that exhibit improved ability to elicit the desired effect on a mammalian immune system.

The selection from large libraries using recursive cycles of reassembly (optionally in combination with other directed evolution methods described herein) to maximally access all the fortuitous but complex mechanisms that cannot be approached rationally

Genetic vaccines can contain a variety of functional components, whose preferred sequences are best determined by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, the empirical sequence evolution described in detail herein. The methods of the invention involve, in general, constructing a separate library for each of the major vector components by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of multiple homologous starting sequences, or other methods of generating a population of recombinants, resulting in a complex mixture of chimeric sequences. The best sequences are selected from these libraries using the high-throughput assays described below. After one or more cycles of selection from each of the single module libraries, the pools of the best sequences of different modules can be combined by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly as long as the screens are compatible. The screens for promoter, enhancer, intron, transfer sequences, mammalian ori, bacterial ori and bacterial marker, and the like, can eventually be combined, resulting in co-optimization of the context of each sequence. An important aspect in these experiments is the selection from large libraries using recursive cycles of reassembly (optionally in combination with other directed evolution methods described herein) to maximally access all the fortuitous but complex mechanisms that cannot be approached rationally, such as DNA transfer into the cell.

**A library of different vectors can be generated by assembling vector modules that provide promoters, cytokines, cytokine antagonists, chemokines, immunostimulatory sequences, and costimulatory molecules using assembly PCR and combinatorial molecular biology**

5

Assembly PCR is a method for assembly of long DNA sequences, such as genes, non-stochastically generated nucleic acid building blocks, and fragments of plasmids. In contrast to PCR, there is no distinction between primers and template, because the non-stochastically generated nucleic acid building blocks &/or fragments to be assembled prime each other. The library of vector modules obtained as described herein can be fused with promoters, which can themselves be optimized by the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention. The resulting genes can be assembled combinatorially into DNA vaccine vectors, where each gene is expressed under a different promoter (e.g., a promoter derived from a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) CMV promoters), and the vector library is screened as described herein to identify vectors which exhibit the desired effect on the immune system.

20

**Properties that influence the efficacy or desirability of the vaccine**

The methods of the invention are useful for obtaining genetic vaccines that are optimized for one or more of many properties that influence the efficacy or desirability of the vaccine. These properties include, but are not limited to, the following.

25



### 2.6.1. EPISOMAL VECTOR MAINTENANCE

**Episomally replicating vectors are maintained in a cell for a longer period of time and permit the development of self-boosting vaccines**

5

One property that one can optimize using the sequence reassembly methods of the invention is the ability of a genetic vaccine vector to replicate episomally in a mammalian cell. Episomal replication of a vaccine vector is advantageous in many situations. For example, episomally replicating vectors are maintained in a cell for a longer period of time than non-replicating vectors, thus resulting in an increased length of immune response modulation or increased delivery of a therapeutically useful protein. Episomal replication also permits the development of self-boosting vaccines which, unlike traditional vaccines, do not require multiple vaccine administrations. For example, a self-boosting vaccine vector can include an antigen-encoding gene which is under the control of an inducible control element which allows induction of antigen expression, and the corresponding immune response, in response to a specific stimulus. However, screening for naturally occurring vector modules which result in enhanced episomal maintenance using traditional approaches or attempts to rationally design mutants with improved properties would require many person-years of research. The invention provides methods for generating and screening orders of magnitude more diversity in a short time period.

10

15

20

Using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to recombine at least two forms of a nucleic acid which is capable of conferring upon a genetic vector the ability to replicate autonomously in mammalian cells

The ability of a genetic vaccine vector to replicate episomally can be optimized by using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to recombine at least two forms of a nucleic acid which is capable of conferring upon a genetic vector the ability to replicate autonomously in mammalian cells. The two or more forms of the episomal replication vector module differ from each other in two or more nucleotides. A library of recombinant episomal replication vector modules is produced, and the library is screened to identify one or more optimized replication vector modules which, when placed in a genetic vaccine vector, confer upon the vector an enhanced ability to replicate autonomously compared to a vector which contains a non-optimized episomal replication vector module.

Repetition of the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly process at least once to identify modules which exhibit enhanced ability to confer episomal maintenance upon a vector containing the module

In one embodiment, the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly process is repeated at least once using as a substrate an optimized episomal replication vector module obtained from a previous round of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly.

The optimized vector module obtained in the earlier round is reassembled (&/or subjected to one or more directed evolution methods described herein) with a further form of the vector module, which can be the same as one of the forms used in the earlier round, or can be a different form of a nucleic acid that functions  
5 as an episomal replication element. Again, a library of recombinant episomal replication vector modules is produced, and the screening process is repeated to identify those episomal replication modules which exhibit enhanced ability to confer episomal maintenance upon a vector containing the module.

10

**Ability to replicate autonomously in eukaryotic cells- examples**

Nucleic acids which are useful as substrates for the use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic  
15 polynucleotide reassembly to optimize episomal replication ability include any nucleic acid that is involved in conferring upon a vector the ability to replicate autonomously in eukaryotic cells. For example, papillomavirus sequences E I and E2, simian virus 40 (SV40) origin of replication, and the like.

20

**Genes from human papillomaviruses are exemplary episomal replication vector modules**

Exemplary episomal replication vector modules that can be optimized using the  
25 methods of the invention are genes from human papillomaviruses (HPV) which are involved in episomal replication. HPV are non-tumorigenic viruses which replicate episomally in skin and are stably expressed *in vivo* for years. Bernard and Apt (1994) Arch. Dermatol. 130: 210.

**Increased episomal maintenance of the HPV genes involved in episomal replication using directed evolution**

5           Despite these *in vivo* properties, it has not been possible to maintain HPV episomally in tissue culture due to underreplication. The invention provides methods by which HPV genes involved in episomal maintenance can be optimized for use in genetic vaccine vectors. HPV genes involved in episomal replication include, for example, the E1 and E2 genes. Thus, according to one  
10   embodiment of the invention, either or both of the HPV E1 and E2 genes are subjected to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to obtain a recombinant episomal replication module which, when placed in a nucleic acid vaccine vector, results in increased maintenance of the vector in mammalian cells. In a preferred  
15   embodiment, the HPV E1 and E2 genes from different, but closely related, benign HPVs are used in a polynucleotide reassembly procedure, as shown, described &/or referenced herein (including incorporated by reference). For example, polynucleotide shuffling of HPV E1 and E2 genes from closely related strains of HPV (such as, for example, HPV 2, 27, and 57) can be used to obtain a library of  
20   recombinant E1 and E2 genes which are then subjected to an appropriate screening method to identify those that exhibit improved episomal maintenance properties.

**Identification, selection, enrichment of recombinant episomal replication**  
25   **vector modules that exhibit improved ability to mediate episomal maintenance**

To identify recombinant episomal replication vector modules that exhibit improved ability to mediate episomal maintenance, members of the library of

- recombinant vector modules are inserted into vectors which are introduced into mammalian cells. The cells are propagated for at least several generations, after which cells that have maintained the vector are identified. Identification can be accomplished, for example, employing a vector that includes a selectable marker.
- 5 Cells containing the library members are propagated in the absence of selection for the selectable marker for at least several generations, after which selective pressure is added. Cells which survive selection are enriched for cells that harbor vectors which contain a recombinant vector module which enhances the ability of the vector to replicate episomally. DNA is recovered from the selected cells and
- 10 introduced into bacterial host cells, allowing recovery of episomal, non-integrated vectors.

**Screening by introducing to a vector containing a polynucleotide encoding an**  
**antigen that is present on the surface of the cell when expressed**

15

- In another embodiment of the invention, the screening step is accomplished by introducing members of the library of recombinant episomal replication vector modules into a vector that includes a polynucleotide that
- 20 encodes an antigen which, when expressed, is present on the surface of a cell. The library of vectors is introduced into mammalian cells which are propagated for at least several generations, after which cells which display the cell surface antigen on the surface of the cell are identified. Such cells most likely harbor a genetic vaccine vector which enhances the ability of the vector to replicate autonomously.

**Use of optimized recombinant episomal replication vector module to construct genetic vaccine vectors**

5           Upon identifying cells which contain an episomally maintained vector, the optimized recombinant episomal replication vector module is obtained and used to construct genetic vaccine vectors. Cell surface antigens which are suitable for use in the screening methods are described above, and others are known to those of skill in the art. Preferably, an antigen is used for which a convenient means of  
10   detection is available.

**Preferred cells for use in the screening methods**

15           Cells which are suitable for use in the screening methods include both cultured mammalian cells and cells which are present in an animal. To screen for recombinant vector modules that are intended for use in humans, the preferred cells for screening purposes are human cells. Generally, initial screening is accomplished in cell culture, where processing of large libraries of experimentally  
20   evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) material is feasible. In a preferred embodiment, cells which display a vector-encoded cell surface antigen on the cell surface are identified by flow cytometry based cell sorting methods, such as fluorescence activated cell sorting. This approach allows very large numbers ( $> 10^7$ ) cells to be evaluated in a single  
25   vial experiment.

**Further testing for durability *in vivo* in an animal model**

Constructs which replicate autonomously in cell culture and give rise to  
5 strong marker gene expression can be further tested for durability *in vivo* in an  
animal model. For example, mouse models for studies of human tissues in mice  
*in vivo* are described herein. Live pieces of human skin are xenotransplanted onto  
the back of SCID mice, allowing screening of the vector libraries for optimal  
properties in human cells *in vivo*. Recursive selection of episomal vectors will  
10 provide strong selection pressure for vectors that remain episomal, yet provide  
high level of gene expression.

**Introducing a genetic vaccine vector into a mammal that has a functional  
15 human immune system and testing for the existence of an immune response  
against the antigen**

In another embodiment, the screening step involves introducing a genetic  
vaccine vector which includes the recombinant episomal replication vector  
20 module, as well as polynucleotide that encodes an antigen or pharmaceutically  
useful protein, into a mammal that has a functional human immune system. The  
animal is then tested for the existence of an immune response against the antigen.  
In a preferred embodiment, the mammals used for such assays are non-human  
mammals that have a functional human immune system. For example, a  
25 functional human immune system can be created in an immunodeficient mouse by  
introducing one or more of a human fetal tissue selected from the group  
consisting of liver, thymus, and bone marrow (Roncarolo et al. (1996) Semin.  
Immunol. 8: 207).

Episomally maintained vectors result in high signal-to-noise ratios upon FACS selection and significantly improve the possibility to recover the plasmids from a small number of selected cells

5

Stable episomal vectors which are obtained using the methods of the invention are useful not only as genetic vaccines, but also are useful tools in other library screening applications. In contrast to randomly integrating and transient vectors, episomally maintained vectors result in high signal-to-noise ratios upon FACS selection, and they also significantly improve the possibility to recover the plasmids from a small number of selected cells.

10

#### 2.6.2. EVOLUTION OF OPTIMIZED PROMOTERS FOR EXPRESSION OF AN ANTIGEN

15

Optimizing the promoter and/or other control sequence to improve the efficacy of genetic vaccinations, reduce the amount of DNA required for protective immunity and thereby the cost of vaccination, control the type of cell in which the gene is expressed, and/or the timing of the antigen expression

20

In another embodiment, the invention provides methods of optimizing vector modules such as promoters and other gene expression control signals. Usually, a coding sequence for an antigen that is delivered by a genetic vaccine is operably linked to an additional sequence, such as a regulatory sequence, to ensure its expression. These regulatory sequences can include one or more of the following: an enhancer, a promoter, a signal peptide sequence, an intron and/or a polyadenylation sequence. A desirable goal is to increase the level of expression

25



of functional expression product relative to that achieved with conventional vectors. The efficacy of a genetic vaccine vector often depends on the level of expression of an antigen by the vaccine vector. An optimized promoter and/or other control sequence is likely to result in improved efficacy of genetic  
5 vaccinations, reduce the amount of DNA required for protective immunity and thereby the cost of vaccination.

Moreover, it is sometimes desirable to have control over the type of cell in which a gene is expressed, and/or the timing of antigen expression. The methods  
10 of the invention provide for optimization of these and other factors which are influenced by promoters and other control sequences.

Improving expression by increasing the rate of production of an expression  
15 product, decreasing the rate of degradation of the expression product, or  
improving the capacity of expression product to perform its intended  
function using stochastic (e.g. polynucleotide shuffling & interrupted  
synthesis) and non-stochastic polynucleotide reassembly of polynucleotides  
involved in control of gene expression

20  
Improved expression of selection markers can be achieved by performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, for example. Expression can effectively be improved by a variety of means, including increasing the rate of production of an  
25 expression product, decreasing the rate of degradation of the expression product or improving the capacity of the expression product to perform its intended function. The methods involve subjecting to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly polynucleotides which are involved in control of gene expression. At least first

and second forms of a nucleic acid that comprises a control sequence, which forms differ from each other in two or more nucleotides, are reassembled (&/or subjected to one or more directed evolution methods described herein) as described above. The resulting library of recombinant transfer modules are  
5 screened to identify at least one optimized recombinant control sequence that exhibits enhanced strength, inducibility, or specificity.

10 **Introduction of the recombinant segments at the level of fragments (non-tochastically generated &/or randomly generated) and *in vitro***

The substrates for reassembly (&/or one or more additional directed evolution methods described herein) can be the full-length vectors, or fragments thereof, which include a coding sequence and/or regulatory sequences to which  
15 the coding sequence is operably linked. The substrates can include variants of any of the regulatory and/or coding sequence(s) present in the vector. If reassembly (&/or one or more additional directed evolution methods described herein) is effected at the level of fragments, the recombinant segments should be reinserted into vectors before screening. If reassembly (&/or one or more additional directed  
20 evolution methods described herein) proceeds *in vitro*, vectors containing the recombinant segments are usually introduced into cells before screening. An example of a vector suitable for use in screening of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) promoters and other regulatory regions is shown, described &/or referenced  
25 herein (including incorporated by reference).

**Using an easily detected selection marker (green fluorescent protein, cell surface protein) when an additional or substitute marker is required**

5           Cells containing the recombinant segments can be screened by detecting expression of the gene encoded by the selection marker. For purposes of selection and/or screening, a gene product expressed from a vector is sometimes an easily detected marker rather than a product having an actual therapeutic purpose, e.g., a green fluorescent protein (see, Crameri (1996) Nature Biotechnol. 14: 315-319) or  
10 a cell surface protein. For example, if this marker is green fluorescent protein, cells with the highest expression levels can be identified by flow cytometry-based cell sorting. If the marker is a cell surface protein, the cells are stained with a reagent having affinity for the protein, such as antibody, and again analyzed by flow cytometry-based cell sorting. However, some genes having a therapeutic  
15 purpose, e.g., drug resistance genes, themselves provide a selectable marker, and no additional or substitute marker is required. Alternatively, the gene product can be a fusion protein comprising any combination of detection and selection markers. Internal reference marker genes can be included on the vector to detect and compensate for variations in copy number or insertion site.

20

**Further round of reassembly (&/or one or more additional directed evolution methods described herein) and screening.**

25           Recombinant segments from the cells showing highest expression of the marker gene can be used as some or all of the substrates in a further round of reassembly (&/or one or more additional directed evolution methods described herein) and screening, if additional improvement is desired.

### 2.6.2.1.CONSTITUTIVE PROMOTERS

Evolving control sequences (promoters, enhancers, etc.) to express a gene of  
5 interest at a higher level than is a gene operably linked to a non-evolved  
control sequences

The invention provides methods of evolving nucleotide sequences that are capable of directing constitutive expression of a gene of interest which is operably  
10 linked to the control sequence. Typically, the control sequences, which can include promoters, enhancers, and the like, are evolved so that a gene of interest is expressed at a higher level than is a gene operably linked to a non-evolved control sequence. To screen for control sequences which are of increased strength, a recombinant library of control sequences can be introduced into a population of  
15 cells and the level of expression of a detectable marker operably linked to the control sequences determined. Preferably, the optimized promoter is capable of expressing an operably linked gene at a level that is at least about 30% greater than that of a control promoter construct, more preferably the optimized promoter is at least about 50% stronger than a control, and most preferably at least about  
20 75% or more stronger than a control promoter.

Using improved CMV promoter/enhancer elements (SV40 and Sra) to  
express foreign genes both in animal models and in clinical applications  
25

Examples of promoters which can be used as substrates in the methods include any constitutive promoter that functions in the intended host cell. The major immediate-early (IE) region transcriptional regulatory elements, including promoter and enhancer sequences (the promoter/enhancer region), of

cytomegalovirus (CMV) is widely used for regulating transcription in vectors used for gene therapy because it is highly active in a broad range of cell types. Optimized CMV transcriptional regulatory elements which direct increased levels of antigen expression is generated by the recursive reassembly (&/or one or more additional directed evolution methods described herein) methods of the invention, resulting in improved efficacy of gene therapy. As the CMV promoter and enhancer is active in human and animal cells, the improved CMV promoter/enhancer elements are used to express foreign genes both in animal models and in clinical applications. Other constitutive promoters that are amenable to use in the claimed methods include, for example, promoters from SV40 and SR , and other promoters known to those of skill in the art.

**Creating a library of chimeric transcriptional regulatory elements through stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of wild-type sequences from two or more of the five related strains of CMV, obtaining the promoter, enhancer and first intron sequences of the IE region through PCR of the CMV strains**

In a preferred embodiment, a library of chimeric transcriptional regulatory elements is created by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of wild-type sequences from two or more of the five related strains of CMV. The promoter, enhancer and first intron sequences of the IE region are obtained by PCR from the CMV strains: human VR-53 8 strain AD169 (Rowe (195 6) Proc. Soc. Exp. Biol. Med. 92:418; human V-977 strain Towne (Plotkin (1975) Infect. Immunol. 12:521-527); rhesus VR-677 strain 68-1 (Asher (1969) Bacteriol. Proc. 269:91); vervet VR-706 strain CSG (Black (1963) Proc. Soc. Exp. Biol. Med. 112:60 1); and, squirrel monkey VR-1398 strain SqSHV (Rangan (1980) Lab. Animal Sci. 30:532). The promoter/

enhancer sequences of the human CMV strains are 95% homologous, and share 70% homology with the sequences of the monkey isolates, allowing the use of polynucleotide reassembly (optionally in combination with other directed evolution methods described herein) to generate a library great diversity.

- 5 Following reassembly (optionally in combination with other directed evolution methods described herein), the library is cloned into a plasmid backbone and used to direct transcription of a marker gene in mammalian cells. An internal marker under the control of a native promoter is typically included in the plasmid vector, which will allow analysis and sorting of cells harboring equal numbers of vectors.

10

- Expression markers, such as green fluorescent protein (GFP) and CD86 (also known as B7.2, see Freeman (1993) J Exp. Med 178:2185, Chen (1994) J Immunol. 152:4929) can also be used. In addition, transfection of SV40 T antigen-transformed cells can be used to amplify a vector which contains an SV40
- 15 origin of replication. The transfected cells are screened by FACS sorting to identify those which express high levels of the marker gene, normalized against the internal marker to account for differences in vector copy numbers per cell. If desired, vectors carrying optimal, recursively reassembled (&/or subjected to one or more directed evolution methods described herein) promoter sequences are
- 20 recovered and subjected to further cycles of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

### 2.6.2.2. CELL-SPECIFIC PROMOTERS

5 Reducing the risk of autoimmune disorder following introduction of foreign  
antigens into host cells and providing for efficient induction of protective  
immunity through the expression of genetic vaccines in professional APCs,  
such as dendritic cells and macrophages

One of the safety concerns associated with genetic vaccines has been the  
10 possibility of autoimmune disorders following introduction of foreign antigens  
into host cells. This risk can be reduced if the pathogen antigen is specifically  
expressed in professional APCs that express the proper costimulatory molecules.  
Although it is somewhat debatable which cells are the most important cells  
expressing the pathogen antigen following genetic vaccinations, it is likely that  
15 professional APCs are involved. It has been shown that blood monocytes express  
antigen following intramuscular injection of genetic vaccine vectors, and dendritic  
cells derived from lymph nodes of vaccinated animals efficiently induced antigen-  
specific T cell activation (C. Bona, The First Gordon Conference on Genetic  
Vaccines, Plymouth, NH, July 21, 1997). These data, together with previous  
20 studies indicating that small number of dendritic cells expressing antigen or  
antigenic peptides is sufficient to induce activation of antigen-specific T cells  
(Thomas and Lipsky, Stem Cells 14: 196, 1996), support the conclusion that  
genetic vaccines specifically expressed in professional APC, such as dendritic  
cells and macrophages, are likely to provide efficient induction of protective  
25 immunity with minimized chance of adverse effects.

**Methods for obtaining promoters and enhancers that induce high expression levels specifically in professional APCs, exploiting natural diversity as a source of substrates for stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly**

The present invention provides methods of obtaining promoters and enhancers that induce high expression levels specifically in professional APCs. Previously existing APC-specific vectors did not provide sufficient expression levels following genetic vaccinations. The methods involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly as described above using as substrates different forms of a nucleic acid that comprises an APC- specific promoter or other control signal. Suitable promoters include, for example, the MHC Class II, and the CD11b, CD11c, and CD40 promoters. Natural diversity of the promoters can be exploited as a highly appropriate source of substrates for the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly. For example, genomic DNA from monkeys, pigs, dogs, cows, cats, rabbits, rats and mice, can be obtained, and the proper sequences obtained by using multiple PCR primers specific for the most conserved regions based on known sequence information. The selection of the optimal promoters can be done in monocytic or B cell lines, such as U937, HL60 or Jijoye, using FACS- sorting. In addition, SV40<sup>+</sup> cell lines, such as COS-1 and COS-7, can be used to improve the recovery of the plasmids. Further analysis can be undertaken in human dendritic cells obtained by culturing peripheral blood monocytes in the presence of IL-4 and GM-CSF as described (Chapuis et al. (1997) Eur. J Immunol. 27: 43 1).



### 2.6.2.3. INDUCIBLE PROMOTERS

5 Using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of two substrates, such as tetracycline and hormone inducible expression systems, to increase the expression level and inducibility *in vivo* of the promoter controlling transgene expression

10 A particularly desirable property of a genetic vaccines would be an ability to induce the promoter controlling transgene expression simply by taking an innocuous oral drug, resulting in a boost of the immune response. Essential requirements for inducible promoters are low base-line expression and strong inducibility. Several promoters with exquisite *in vitro* regulation exist, but the  
15 expression level and inducibility of each is too low to be useable *in vivo*. The invention overcome these problems by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly using as substrates two or more variants of a nucleic acid that functions as an inducible control sequence. Suitable substrates include, for example, tetracycline and  
20 hormone inducible expression systems, and the like. Hormones that have been used to regulate gene expression include, for example, estrogen, tomosifen, toremifen and ecdysone (Ramkumar and Adler (1995) Endocrinology 136: 536-542). Libraries of recombinant inducible promoters are screened as described above in the presence and absence of the inducer.

25

**Tetracycline responsive system provides possibilities to induce and turn off gene expression (ecdysone responsive element another candidate)**

5           The most commonly used inducible gene expression protocol is the tetracycline responsive system, which provides possibilities to both induce and turn off gene expression (Gossen and Bujard (1992) Proc. Nat'l. Acad. Sci. USA 89: 5547; Gossen et al. (1995) Science 268: 1766). A repressor gene is located on the plasmid and binds to an operator in the promoter. Tetracycline or doxycycline  
10 modulates the binding ability of the repressor. Interestingly, four amino acid changes convert the repressor into an activator. In addition to the tetracycline responsive system, other candidates for inducible promoter evolution include the ecdysone responsive element (No et al., Proc. Nat'l. Acad. Sci. USA 93:3346,1997).

15

**Inducible promoters provide a means by which a vaccine dose can be administered subsequent to the initial administration simply by ingestion of a reagent that causes induction of the inducible promoter**

20

          Inducible promoters such as those obtained using the methods of the invention are useful in autoboot vaccines. Particularly when combined with a stably maintained episomal vector obtained as described above, the inducible promoters provide a means by which a vaccine dose can be administered  
25 subsequent to the initial administration simply by ingestion of a reagent that causes induction of the inducible promoter. A flow cytometry-based screening protocol that is suitable for optimization of inducible promoters is diagrammed herein.

**Testing the functionality of autobosting vaccines in a mouse model**

5       The functionality of autobosting vaccines can be tested in a mouse model  
such as that described above. Genetic vaccine vectors are injected into the skin of  
normal mice and into human skin in SCID-human skin mice. A gene encoding  
hepatitis B surface antigen (HBsAg) or other surface antigen is incorporated into  
these vectors enabling direct measurements of the levels of antigen produced,  
because HBsAg levels can be measured in cell culture supernates and in the  
10   circulation of the mice. The drug inducing the expression of the antigen is given  
after 1, 2, 4 and 6 weeks, and the expression levels of HBsAg are studied.  
Moreover, the levels of anti-HBsAg antibodies are measured. The mice are also  
injected with a vector containing a pathogen antigen discovered by ELI, and  
specific immune responses are followed.

15

**In vivo assessment of functionality of autobosting genetic vaccines in human  
immune system using SCID-human skin model with SCID-hu mouse model**

20       Combining the SCID-human skin model with traditional SCID-hu mouse  
model (Roncarolo et al., Semin. Immunol. 8: 207, 1996) allows the assessment of  
functionality of autobosting genetic vaccines in human immune system *in vivo*,  
and also allows measurements of human Ab responses *in vivo*. This model can  
also be used to assess production of HBsAg after oral boosting of novel genetic  
25   vaccine vectors harboring the gene encoding HBsAg.

### 2.6.3. EVOLUTION OF BINDING POLYPEPTIDES THAT ENHANCE SPECIFICITY AND EFFICIENCY OF GENETIC VACCINES

5           The present invention also provides methods for obtaining recombinant nucleic acids that encode polypeptides which can enhance the ability of genetic vaccines to enter target cells. Although the mechanisms involved in DNA uptake are not well understood, the methods of the invention enable one to obtain genetic vaccines that exhibit enhanced entry to cells, and to appropriate cellular  
10   compartments.

Enhancing the efficiency and specificity of a genetic vaccine nucleic acid uptake by a given cell type by coating the nucleic acid with an evolved protein that binds to the genetic vaccine nucleic acid, and is also capable of  
15 binding to the target cell

          In one embodiment, the invention provides methods of enhancing the efficiency and specificity of a genetic vaccine nucleic acid uptake by a given cell  
20   type by coating the nucleic acid with an evolved protein that binds to the genetic vaccine nucleic acid, and is also capable of binding to the target cell. The vector can be contacted with the protein *in vitro* or *in vivo*. In the latter situation, the protein is expressed in cells containing the vector, optionally from a coding sequence within the vector. The nucleic acid binding proteins to be evolved  
25   usually have nucleic acid binding activity but do not necessarily have any known capacity to enhance or alter nucleic acid DNA uptake.

**DNA binding proteins that can be used in these methods**

5 DNA binding proteins which can be used in these methods include, but are not limited to, transcriptional regulators, enzymes involved in DNA replication (e.g., *recA*) and reassembly (&/or one or more additional directed evolution methods described herein), and proteins that serve structural functions on DNA (e.g., histones, protamines). Other DNA binding proteins that can be used include  
10 the phage 434 repressor, the lambda phage *ci* and *cro* repressors, the E. coli CAP protein, myc, proteins with leucine zippers and DNA binding basic domains such as *fos* and *jun*; proteins with 'POU' domains such as the *Drosophila* paired protein; proteins with domains whose structures depend on metal ion chelation such as Cys<sub>2</sub>His<sub>2</sub> zinc fingers found in TFIID, Zn<sub>2</sub>(Cys)<sub>6</sub> clusters such as those found in  
15 yeast *Gal4*, the Cys<sub>3</sub> His box found in retroviral nucleocapsid proteins, and the Zn<sub>2</sub>(Cys)<sub>8</sub> clusters found in nuclear hormone receptor-type proteins; the phage P22 Arc and Mnt repressors (see Knight et al. (1989) J Biol. Chem. 264: 3639-3642 and Bowie & Sauerkl 1989) J Biol. Chem. 264: 7596-7602. RNA binding proteins are reviewed by Burd & Dreyfuss (1994) Science 265: 615-621, and  
20 include HIV Tat and Rev.

**Formats for performing reassembly (&/or one or more additional directed evolution methods described herein)**

25

As in other methods of the invention, evolution of DNA binding proteins toward acquisition of improved or altered uptake efficiency is effective by one or more cycles of reassembly (&/or one or more additional directed evolution methods described herein) and screening. The starting substrates can be nucleic

acid segments encoding natural or induced variants of one or nucleic acid binding proteins, such as those mentioned above. The nucleic acid segments can be present in vectors or in isolated form for the reassembly (&/or one or more additional directed evolution methods described herein) step. reassembly (&/or  
5 one or more additional directed evolution methods described herein) can proceed through any of the formats described herein.

For screening purposes, the reassembled (&/or subjected to one or more directed evolution methods described herein) nucleic acid segments are typically  
10 inserted into a vector, if not already present in such a vector during the reassembly (&/or one or more additional directed evolution methods described herein) step.

**Including binding site in vector for DNA binding protein recognizing a**  
15 **specific binding site**

The vector generally encodes a selective marker capable of being expressed in the cell type for which uptake is desired. If the DNA binding protein being evolved recognizes a specific binding site (*e.g.*, *lacI* binding protein  
20 recognizes *lacO*), this binding site can be included in the vector. Optionally, the vector can contain multiple binding sites in tandem.

**Transforming vectors containing recombinant segments into host cells and**  
25 **lysing cells under mild conditions that do not disrupt binding of vectors to**  
**DNA binding proteins**

The vectors containing different recombinant segments are transformed into host cells, usually *E. coli*, to allow recombinant proteins to be expressed and

bind to the vector encoding their genetic material. Most cells take up only a single vector and so transformation results in a population of cells, most of which contain a single species of vector. After an appropriate period to allow for expression and binding, cells are lysed under mild conditions that do not disrupt binding of vectors to DNA binding proteins. For example, a lysis buffer of 35mM HEPES (pH 7.5 with KOH), 0.1mM EDTA, 100mM Na glutamate, 5% glycerol, 0.3mg/ml BSA, 1mM DTT, and 0.1mM PMSF) plus lysozyme (0.3-ml at 10 mg/ml) is suitable (see Schatz et al., US 5,338,665). The complexes of vector and nucleic acid binding protein are then contacted with cells of the type for which improved or altered uptake is desired under conditions favoring uptake. Suitable recipient cells include the human cell types that are common targets in DNA vaccination. These cells include muscle cells, monocytes/macrophages, dendritic cells, B cells, Langerhans cells, keratinocytes, and the M-cells of the gut. Cells from mammals including, for example, human, mouse, and monkey can be used for screening. Both primary cells and cells obtained from cell lines are suitable.

**Recovery of cells expressing marker and enriching for recombinant segments for further rounds of selection**

20

After incubation, cells are plated with selection for expression of the selective marker present in the vector containing the recombinant segments. Cells expressing the marker are recovered. These cells are enriched for recombinant segments encoding nucleic acid binding proteins that enhance uptake of vectors encoding the respective recombinant segments. The recombinant segments from cells expressing the marker can then be subjected to a further round of selection. Usually, the recombinant segments are first recovered from cells, e.g., by PCR amplification or by recovery of the entire vectors. The recombinant segments can then be reassembled (&/or subjected to one or more directed evolution methods

described herein) with each other or with other sources of DNA binding protein variants to generate further recombinant segments. The further recombinant segments are screened in the same manner as before.

5

Using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to evolve, particularly, the carboxy- and amino-terminal peptide extensions of the histone protein, to increase the efficiency of DNA transfer into the cells

10

One example of a method to evolve an optimized nucleic acid binding domain involves the reassembly (optionally in combination with other directed evolution methods described herein) of histone genes. Histone-condensed DNA can result in increased gene transfer into cells. See, e.g., Fritz et al. (1996) Human Gene Therapy 7: 1395-1404. Thus, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be used to evolve the histone protein, particularly the carboxy- and amino-terminal peptide extensions, to increase the efficiency of DNA transfer into cells. In this approach, the histone is encoded by the DNA to which it will be bound.

20

#### Construction of the histone library

The histone library can be constructed by, for example, 1) reassembly (optionally in combination with other directed evolution methods described herein) of many related histone genes from natural diversity, 2) addition of random or partially randomized peptide sequences at the N- and C-terminal sequences of the histone, 3) by addition of pre-selected protein-encoding regions to the N- or C-termini, such as whole cDNA libraries, nuclear protein ligand

25



libraries, etc. These proteins can be partially randomized and linked to the histone by a library of linkers.

- 5    Starting substrates for evolving nucleic acid binding sites contain variant binding sites and recombinant forms of these sites are screened as a component of a vector that also encodes a nucleic acid binding protein

10    In a variation of the above procedure, a binding site recognized by a nucleic acid binding protein can be evolved instead of, or as well as, the nucleic acid binding protein. Nucleic acid binding sites are evolved by an analogous procedure to nucleic acid binding proteins except that the starting substrates contain variant binding sites and recombinant forms of these sites are screened as a component of a vector that also encodes a nucleic acid binding protein.

15

- When the evolved DNA binding protein does not have a high degree of sequence specificity and it is unknown precisely which sites of the vector used in screening are bound by the protein, the vector should include all or most  
20 of the screening vector sequences together with additional sequences required to effect vaccination or therapy

25    Evolved nucleic acid segments encoding DNA binding proteins and/or evolved DNA binding sites can be included in genetic vaccine vectors. If the affinity of the DNA binding protein is specific to a known DNA binding site, it is sufficient to include that binding site and the sequence encoding the DNA binding protein in the genetic vaccine vector together with such other coding and regulatory sequences are required to effect gene therapy. In some instances, the evolved DNA binding protein may not have a high degree of sequence specificity

and it may be unknown precisely which sites on the vector used in screening are bound by the protein. In these circumstances, the vector should include all or most of the screening vector sequences together with additional sequences required to effect vaccination or therapy. An exemplary selection scheme which employs M  
5 13 protein VIII is shown, described &/or referenced herein (including incorporated by reference).

**Target cells of interest**

10

Target cells of interest include, for example, muscle cells, monocytes, dendritic cells, B cells, Langerhans cells, keratinocytes, M-cells of the gut, and the like. Cell- specific ligands that are suitable for use with each of the cell types are known to those of skill in the art. For example, suitable proteins to direct  
15 binding to antigen presenting cells include CD2, CD28, CTLA-4, CD40 ligand, fibrinogen, factor X, ICAM-1, - glycan (zymosan), and the Fc portion of immunoglobulin G. (Weir's Handbook of Experimental Immunology, Eds. L.A. Herzenberg, D.M. Weir, L.A. Herzenberg, C. Blackwell, 5th edition, volume IV, chapters 156 and 174) because their respective ligands are present on APCs,  
20 including dendritic cells, monocytes/macrophages, B cells, and Langerhans cells. Bacterial enterotoxins or subunits thereof are also of interest for targeting purposes.

**LPS facilitates the interaction between vector and monocytes and is also likely to act as an adjuvant, further potentiating the immune responses**

- 5           The ability of the vectors to enter and activate APC, such as monocytes, can also be enhanced by coating the vectors with small quantities of lipopolysaccharide (LPS). This facilitates the interaction between vector and monocytes, which have a cell surface receptor for LPS. Due to its immunostimulatory activities, LPS is also
- 10   likely to act as an adjuvant, thereby further potentiating the immune responses.

**Receptor binding components of enterotoxins can be evolved for improved attachment to cell surface receptors, improved entry to and transport across the cells of the intestinal epithelium, and improved binding to, and activation of, B cells or other APCs**

15

- Enterotoxins produced by certain pathogenic bacteria are useful as agents that bind cells and thus enhance delivery of vaccines, antigens, gene therapy
- 20   vectors and pharmaceutical proteins. In an exemplary embodiment of the invention, receptor binding components of enterotoxins derived from *Vibrio cholerae* and enterotoxigenic strains of *E. coli* are evolved for improved attachment to cell surface receptors and for improved entry to and transport across the cells of the intestinal epithelium. In addition, they can be evolved for
- 25   improved binding to, and activation of, B cells or other APCs. An antigen of interest can be fused to these toxin subunits to illustrate the feasibility of the approach in oral delivery of proteins and to facilitate the screening of evolved enterotoxin subunits. Examples of such antigens include growth hormone, insulin, myelin basic protein, collagen and viral envelope proteins.

Vectors that contain the library of recombinant enterotoxin binding moiety nucleic acids are transfected into a population of host cells, wherein the recombinant enterotoxin binding moiety nucleic acids are expressed to form recombinant enterotoxin binding moiety polypeptides

These methods involve reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprises a polynucleotide that encodes a preferably non-toxic receptor binding moiety of an enterotoxin. The first and second forms differ from each other in two or more nucleotides, so the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly results in production of a library of recombinant enterotoxin binding moiety nucleic acids. Suitable enterotoxins include, for example, a V cholerae enterotoxin, enterotoxins from enterotoxigenic strains of E. coli, salmonella toxin, shigella toxin and campylobacter toxin. Vectors that contain the library of recombinant enterotoxin binding moiety nucleic acids are transfected into a population of host cells, wherein the recombinant enterotoxin binding moiety nucleic acids are expressed to form recombinant enterotoxin binding moiety polypeptides. In a preferred embodiment, the recombinant enterotoxin binding moiety polypeptides are expressed as fusion proteins on the surface of bacteriophage particles. The recombinant enterotoxin binding moiety polypeptides can be screened by contacting the library with a cell surface receptor of a target cell and determining which recombinant enterotoxin binding moiety polypeptides exhibit enhanced ability to bind to the target cell receptor. The cell surface receptor can be present on the surface of a target cell itself, or can be attached to a different cell, or binding can be tested using cell surface receptor that is not associated with a cell. Examples of suitable cell surface receptors include,

for example, Gm I. Similarly, one can evolve bacterial superantigens for altered (increased or decreased) binding to T cell receptor and MHC class II molecules. These superantigens activate T cells in an antigen nonspecific manner.

- 5 Superantigens binding to T cell receptor/MHC class II molecules include Staphylococcal enterotoxin B, *Urtica dioica* superantigen (Musette et al. (1996) Eur. J Immunol. 26:618- 22) and Staphylococcal enterotoxin A (Bavari et al. (1996) J Infect. Dis. 174:338-45). Phage display has been shown to be effective when selecting superantigens that bind MHC class II molecules (Wung and  
10 Gascoigne (1997) J Immunol. Methods. 204:33- 41).

**Both CT and CT-B have been shown to have potent adjuvant activities *in vivo* and they enhance immune responses after oral delivery of antigens and**  
15 **vaccines**

- Cholera toxin (CT) is an oligomeric protein of 84,000 daltons which consists of one toxic A subunit (CT-A) covalently linked to five B subunits (CT-B). CT-B functions as the-receptor binding component and binds to GM<sub>1</sub>,  
20 ganglioside receptors on mammalian cell surfaces. The toxic A-subunit is not necessary for the function of CT, and in the absence of CT-A, functional CT-B pentamers can form (Lebens and Holingren (1994) Dev. Biol. Stand. 82: 215-227). Both CT and CT-B have been shown to have potent adjuvant activities *in vivo* and they enhance immune responses after oral delivery of antigens and  
25 vaccines (Czerkinsky et al. (1996) Ann. NY Acad. Sci. 778: 185-93; Van Cott et al. (1996) Vaccine 14: 392-8). Moreover, a single dose of CT-B conjugated to myelin basic protein prevented onset of autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (Czerkinsky et al., supra.). Furthermore, feeding animals with myelin basic protein conjugated to CT-B after the onset of

clinical symptoms (7 days) attenuated the symptoms in these animals. Other bacterial toxins, such as enterotoxins of E. coli, Salmonella toxin, Shigella toxin and Campylobacter toxin, have structural similarities with CT. Enterotoxins of E. coli have the same A-B structure as CT and they also have sequence homology and share functional similarities.

**Family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is feasible among enterotoxin-encoding nucleic acids from different bacterial species**

Bacterial enterotoxins can be evolved for improved affinity and entry to cells by polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein). The similarity of E. coli-derived enterotoxin subunit and CT-B is 78%, and several completely conserved regions of more than eight nucleotides can be found. B subunits from two different strains of E. coli are 98% homologous both at sequence and protein levels. Thus, family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is feasible among enterotoxin-encoding nucleic acids from different bacterial species.

**Screen the secretion of chimeric proteins by V cholerae by culturing the bacteria in agar in the presence of monoclonal antibodies specific for the antigen that was fused to the toxins**

The libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) toxin subunits can be

expressed in a suitable host cell, such as *V cholerae*. For safety reasons, strains in which the toxic CT-A is deleted are preferred. An antigen of interest can be fused to the receptor-binding subunit. Secretion of chimeric proteins by *V cholerae* can be screened by culturing the bacteria in agar in the presence of monoclonal  
5 antibodies specific for the antigen that was fused to the toxins and the level of secretion is detected as immunoprecipitation in the agar around the colonies.

10 **Evolving for improved binding to the G<sub>M1</sub>, ganglioside receptor and other receptors, detecting binding between receptor and chimeric fusion proteins with a monoclonal antibody specific for the antigen that was fused to the toxin**

One can also add G<sub>M1</sub>, ganglioside receptors to the agar in order to detect  
15 colonies secreting functional enterotoxin subunits. Colonies producing significant levels of the fusion protein are then cultured in 96-well plates, and the culture medium is tested for the presence of molecules capable of binding to cells or receptors in solution. Binding of chimeric fusion proteins to G<sub>M1</sub>, ganglioside receptors on cell surface or in solution can be detected by a monoclonal antibody  
20 specific for the antigen that was fused to the toxin. The assay using whole cells has the advantage that one may evolve for improved binding also to receptors other than the G<sub>M1</sub>, ganglioside receptor. When increasing concentrations of wild-type enterotoxins are added to these assays, one can detect mutants that bind to receptors with improved affinities. Affinity and specificity of toxin binding can  
25 also be determined by surface plasmon resonance (Kuziemko et al. (1996) Biochemistry 35: 6375- 84).

**Advantage of large scale production and avoidance of problems  
associated with expression on phage in the bacterial expression system**

5 The advantage of the bacterial expression system is that the fusion protein  
is secreted by bacteria that could potentially be used in large scale production.  
Moreover, because the fusion protein is in solution during selection, possible  
problems associated with expression on phage (such as bias towards selection of  
mutants that only function on phage) can be avoided.

10

**In phage display, mutants can be easily further selected in *in vivo*  
assays when screening to identify enterotoxins with improved affinities**

15 Nevertheless, phage display is useful for screening to identify enterotoxins  
with improved affinities. A library of experimentally evolved (e.g. by  
polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis)  
mutants can be expressed on phage, such as M 13, and mutants with improved  
affinity are selected based on binding to, for example, G<sub>M1</sub> ganglioside receptors  
in solution or on a cell surface. The advantage of this approach is that the mutants  
20 can be easily further selected in *in vivo* assays as discussed below. A screening  
approach using fusion to M 13 protein VIII is diagrammed herein.

25 **The recombinant binding moiety is expressed in the cells and binds to  
the nucleic acid binding domain to form a vector-binding moiety complex**

Finally, the resulting evolved enterotoxin can be fused with DNA binding  
protein, and genetic vaccine vectors are coated with this fusion protein. The  
stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-



stochastic polynucleotide reassembly can be done either separately, in which case the two domains are assembled after reassembly (optionally in combination with other directed evolution methods described herein), or in a combined reaction. reassembly (optionally in combination with other directed evolution methods

5 described herein) results in production of a library of recombinant binding moiety nucleic acids which can be screened by transfecting vectors which contain the library, as well as a binding site specific for the nucleic acid binding domain, into a population of host cells. The binding moiety is expressed in the cells and binds to the nucleic acid binding domain to form a vector-binding moiety complex.

10 Host cells can then be lysed under conditions that do not disrupt binding of the vector- binding moiety complex.

**Optimized recombinant binding moiety nucleic acids are isolated from cells**

15 **containing the vector**

The vector -binding moiety complex can then be contacted with a cell of interest, after which cells are identified that contain a vector and the optimized recombinant binding moiety nucleic acids are isolated from the cells.

20

**Increasing the number of copies of target DNA taken into those cells that initially take up the same DNA (mammalian cells)**

25 Another method for obtaining enhanced uptake of a target DNA by mammalian cells is also provided by the invention. Specifically, the method increases the number of copies of target DNA taken into those cells that initially take up the same DNA.

Cells that take up the target molecule of DNA (cell surface expression of membrane-associated DNA binding domains) will express the factor and have increased specific affinity for target DNA that remains extracellular,  
5 while cells that did not take up DNA will be at a competitive disadvantage as they will not bear the cell surface target DNA- specific binding domain,  
which is required for specifically mediated DNA uptake

The method uses cell surface expression of membrane-associated DNA  
10 binding domains of, for example, transcription factors, that are encoded in the target DNA sequence, which also includes the cognate recognition sequence for the binding domain. Uptake of one molecule of target DNA into a cell (by any process, passive uptake, electroporation, osmotic shock, other stress) will lead to transcription of the gene encoding the polynucleotide binding domain. The gene  
15 encoding the binding domain is engineered so that the binding domain is expressed in a membrane anchored form. For example, a hydrophobic stretch of amino acids can be encoded at the carboxyl terminus of the binding domain, thus leading to phospho-inositol-glycan (PIG) conjugation after partial cleavage of this terminal sequence. This, in turn, leads to trafficking and positioning of the  
20 binding domain on the cell surface. The same cells that took up the first molecule of DNA will express the factor and have increased specific affinity for target DNA that remains extracellular. Cells that did not take up DNA will be at a competitive disadvantage as they will not bear the cell surface target DNA-specific binding domain, which is required for specifically mediated DNA uptake.  
25

Enhanced binding of the target DNA to the target cell will increase the efficiency of DNA internalization and desired intracellular function. This process represents a positive feedback for increased DNA uptake into cells that take up DNA first.

**Practical means for determining which transcription factor or combination of factors to use with any particular target DNA**

5

The target DNA, whether a circular or linear plasmid, oligonucleotide, bacterial or mammalian chromosomal fragment, is engineered to bear one or more copies of a DNA recognition sequence for a mammalian or bacterial transcription factor. Many target sequences will already bear one or more such motifs; these  
10 can be identified by sequence analysis. Endogenous motifs recognized by these factors also can be identified experimentally by demonstrating that the target DNA binds to one or more of a panel of transcription factors in an appropriate assay format. This provides a practical means for determining which factor or combination of factors to use with any particular target DNA.

15

**Motif(s) in the case of a small oligonucleotide or a DNA plasmid and in the cases where more than one DNA binding protein will be expressed on the cell surface**

20

In the case of a small oligonucleotide or a DNA plasmid (such as used for a DNA vaccine), appropriate motifs can be engineered into the sequence. A particular motif can be engineered in one or more copies, in tandem or dispersed in the target sequence. Alternatively, a set of different motifs can be engineered,  
25 in tandem or separated, in cases where more than one DNA binding protein will be expressed on the cell surface.

#### 2.6.4. EVOLUTION OF BACTERIOPHAGE VECTORS

5    Using stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and  
  non-stochastic polynucleotide reassembly, phage genetics and display  
  technologies to rapidly evolve highly novel, potent, and generic vaccine  
  vehicles

10    The invention provides methods of obtaining bacteriophage vectors that  
  exhibit desirable properties for use as genetic vaccine vectors. The principle  
  behind the approach provided by the invention is to combine the power of  
  stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-  
  stochastic polynucleotide reassembly with the extraordinary power of  
  bacteriophage genetics and the wealth of recent advances in phage display  
15    technologies to rapidly evolve highly novel, potent, and generic vaccine vehicles.

20    Methods for delivery of antigens from pathogens to professional APCs,  
  maximizing efficiency through increasing the kinetics and potency of the  
  immune response to the vaccine

25    The evolved vaccine vehicles can present antigen either (1) in native form  
  on the surface of these APCs for the induction of an antibody response or (2)  
  selectively invade APCs and deliver DNA vaccine constructs to APCs for  
  intracellular expression, processing and presentation to CTLs. More efficient  
  methods for delivery of antigens from pathogens to professional APCs will  
  increase the kinetics and potency of the immune response to the vaccine.

Affinity maturation process, essential for the generation of antibodies with sufficient affinity to neutralize pathogenic antigens, occurs in germinal centers (spleen) where follicular dendritic cells present protein antigens to B cells and processed antigen fragments to T cells, making efficient delivery of  
5 antigens to FDCs essential in increasing the kinetics and potency of the immune response to the immunizing antigen

Genetic vaccine delivery vehicles that are evolved according to the methods of the invention are particularly valuable for the rapid induction of high  
10 affinity antibodies which can effectively neutralize viral epitopes or pathogenic toxins such as superantigens or cholera toxin. High affinity antibodies are generated by somatic mutation of low affinity primary response antibodies. This so-called affinity maturation process is essential for the generation of antibodies with sufficient affinity to neutralize pathogenic antigens. Affinity maturation  
15 occurs in the spleen in germinal centers where follicular dendritic cells (FDCs), professional antigen presenting cells, present protein antigens to B cells and processed antigen fragments to T cells. Clonally expanding B cell populations which have undergone somatic mutation are selected for those mutant B cells expressing antibodies with improved affinity for antigen. Thus, efficient delivery  
20 of antigen to FDCs will increase the kinetics and potency of the immune response to the immunizing antigen. Additionally, processed antigen bound to MHC is required to stimulate antigen specific T cells. Genetic vaccines are particularly efficient at priming class I MHC restricted responses due to intracellular expression of antigen, with a resultant trafficking of antigen fragments to the class  
25 I MHC pathway. Thus, invasive bacteriophage vectors capable of delivery of genetic vaccine constructs or protein antigens to FDCs are useful.

Preferred bacteriophage for the purpose of evolution are those that have been genetically well characterized and developed for the display of foreign protein epitopes (of special note was M13 bacteriophage, a small filamentous phage which is a versatile, highly evolvable vehicle for efficient and targeted delivery of protein or DNA vaccine vehicles to cellular targets of interest

Any of several bacteriophage can be evolved according to the methods of the invention. Preferred bacteriophage for these purposes are those that have been genetically well characterized and developed for the display of foreign protein epitopes; these include, for example, lambda, T7, and M13 bacteriophage. The filamentous phage M13 is a particularly preferred vector for use in the methods of the invention. M 13 is a small filamentous bacteriophage that has been used widely to display polypeptide fragments in functional, folded form on the surface of bacteriophage particles. Polypeptides have been fused to both the gene III and gene VIII coat proteins for such display purposes. Thus, M13 is a versatile, highly evolvable vehicle for efficient and targeted delivery of protein or DNA vaccine vehicles to cellular targets of interest.

Improvements in methods (efficient delivery of phage, homing to APCs, and invasion of target cells using experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) bacterial invasion proteins) exemplified for bacteriophage vectors and applicable to other types of genetic vaccine vectors

The following three properties are examples of the type of improvements that can be achieved by use of the methods of the invention to evolve bacteriophage genetic vaccine vectors: (1) efficient delivery of phage to the bloodstream by inhalation or oral delivery, (2) efficient homing to APCs, and (3)

efficient invasion of target cells using experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) bacterial invasion proteins. Where M13 is used, fusions can be made to both gene 111 and gene VIII coat proteins so that two evolved properties can be combined  
5 into a single phage particle. These studies can be performed in test animals such as laboratory mice so that the evolved constructs can be rapidly characterized with respect to their potency as vaccine vehicles. Evolved inhalable and/or orally deliverable vehicles and evolved invasins will translate directly for use in human cells, while the principles developed in evolving the ability to home to test animal  
10 APCs are readily transferable to human cells by performing analogous selections on human APCs. While these methods are exemplified for bacteriophage vectors, the methods are also applicable to other types of genetic vaccine vectors.

15       **2.6.4.1.   EVOLUTION OF EFFICIENT DELIVERY OF  
BACTERIOPHAGE VEHICLES BY INHALATION OR ORAL  
DELIVERY**

Method for the formulation of proteins into inhalable colloids that can be  
20 absorbed into the blood stream through the lung (preparation involved in the  
invention)

The invention provides methods for obtaining genetic vaccine vectors that are capable of efficient delivery to the bloodstream upon administration by  
25 inhalation or by oral administration. Methods have been developed for the formulation of proteins into inhalable colloids that can be absorbed into the blood stream through the lung. The mechanisms by which proteins are transported into the blood stream are not clearly understood, and thus improvements are readily approached by evolutionary methods. Using M 13 as an example, the invention

involves preparation of a library of, for example, peptide ligands, adhesion molecules, bacterial enterotoxins, and randomly fragmented cDNA, which are fused to gene 111, for example, of M13. Libraries of  $>10^{10}$  individual fusions are readily achievable with this technology.

5

M13 phage enters the blood stream, can be recovered and amplified in E. coli cells, pass through several rounds of enrichment, and be further characterized and evolved by sequencing and reassembling (optionally in combination with other directed evolution methods described herein) the entire phage genome and subjecting the phage to reiterated cycles of delivery, recovery, amplification, and reassembly (optionally in combination with other directed evolution methods described herein)

15           Screening involves preparation of high titer stocks (preferably  $>10^{12}$  phage particles) in standard colloidal formulations which are delivered intranasally to test animals, such as mice. Blood samples are taken over the course of the ensuing day and circulating phage are amplified in E. coli. It has been established that M13 circulates for long periods in the blood after injection intravenously, and thus

20   it is reasonable to expect that phage that successfully enter the blood stream through the lung can be efficiently recovered and amplified E. coli cells. In a preferred embodiment, several rounds of enrichment are applied to the initial libraries in order to enrich for phage that can efficiently enter the blood stream when delivered intranasally. Candidate clones are typically tested individually for

25   their relative efficiency of entry, and the best clones can be further characterized by sequencing to identify the nature of the fusions that confer efficient delivery (of particular interest from the cDNA libraries). Selected clones can be further evolved and for improved entry by reassembling (optionally in combination with other directed evolution methods described herein) the entire phage genome and



subjecting the phage to reiterated cycles of delivery, recovery, amplification, and reassembly (optionally in combination with other directed evolution methods described herein).

5

To obtain vaccine vectors that are effective when taken orally, recombinant vectors prepared through reassembly (optionally in combination with other directed evolution methods described herein) are administered, surviving, stable vectors are recovered from the stomach, and vectors that efficiently enter the bloodstream and/or lymphatic tissue can be recovered from the blood/lymph.

An analogous procedure is used to obtain vaccine vectors that are effective when delivered orally. A genetic vaccine vector library is prepared by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly. The recombinant vectors are packaged and administered to a test animal. Vectors that are stable in the stomach/intestinal environment are recovered, for example, by recovering surviving vectors from the stomach. Vectors that efficiently enter the bloodstream and/or lymphatic tissue can be identified by recovering vectors that reach the blood/lymph. A schematic of this selection method is shown, described &/or referenced herein (including incorporated by reference).

#### 2.6.4.2. EVOLUTION OF BACTERIOPHAGE VEHICLES FOR EFFICIENT HOMING TO APCs

- 5 Two selection formats: the first consisting of enriching the libraries of random peptide ligands and cDNAs used in (A) above for phage which selectively bind to APCs and using either negative or positive selection; the second consists of injecting phage libraries intravenously, collecting target organs of interest, liberating the phage by sonication, further amplifying and
- 10 enriching.
- The invention also provides methods of evolving bacteriophage vectors, as well as other types of genetic vaccine vectors, for efficient homing to professional antigen presenting cells. Libraries of random peptide ligands and cDNAs used in (A) above are enriched for phage which selectively bind to APCs by first negatively
- 15 selecting for binding to non-APC cell types, and then positively selecting for binding to APCs. The selection is typically performed by mixing high titer stocks of phage from the libraries ( $>10^{12}$  phage particles) with cells ( $\sim 10^7$  cells per selection cycle) and either taking the nonbinding phage (negative selection) or the binding phage from cell pellets (positive selection). An alternative selection
- 20 format consists of injecting phage libraries intravenously, allowing the libraries to circulate for several hours, collecting target organs of interest (lymph node, spleen), and liberating the phage by sonication. The positively selected phage can be amplified in *E. coli* and further rounds of enrichment are performed (3- 5 rounds) if further optimization is desired. After the chosen number of rounds,
- 25 individual phage are characterized for their ability to home to lymphoid organs. The best few candidates can be subjected to further evolution through iterated rounds of selection, amplification, and reassembly (optionally in combination with other directed evolution methods described herein).

### 2.6.4.3. EVOLUTION OF BACTERIOPHAGE FOR INVASION OF APCs

5 The methods of the invention are also useful for evolving bacteriophage and other genetic vaccine vehicles for invasion of target cells. This opens up the possibility of targeting the class I MHC antigen processing pathways with either internalized protein antigen or antigen expressed by DNA vaccine vehicles carried in by the evolved vector.

10

#### Efficient internalization of pathogenic bacteria through invasin interaction with integrins

15 Invasins comprise a large family of bacterial proteins which interact with integrins and promote the efficient internalization of pathogenic bacteria such as Salmonella.

20 Reassembly (optionally in combination with other directed evolution methods described herein) of different forms of polynucleotides encoding invasins, cloning as fusions to the M13 gene VIII coat protein gene, preparing libraries and mixing these libraries with target APCs

25 This embodiment of the invention involves reassembling (optionally in combination with other directed evolution methods described herein) different forms of polynucleotides that encode invasins. For example, two or more genes which encode the invasin family of proteins can be experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis). The experimentally evolved (e.g. by polynucleotide reassembly &/or

polynucleotide site-saturation mutagenesis) polynucleotides can be cloned as fusions to the M 13 gene VIII coat protein gene, for example, and high titer stock of such libraries will be prepared. These libraries of bacteriophage can be mixed with target APCs.

5

**Removing free phage and phage bound to the cell surface**

After incubation, the cells are exhaustively washed to remove free phage and phage bound to the surface of the cells can be removed by panning against polyclonal anti-M13 antibodies.

**Obtaining successful phage, amplifying, reassembling (optionally in combination with other directed evolution methods described herein), and selecting, characterizing for relative invasiveness, combining with gene III fusions (encoding pathogenic epitopes of interest) and testing for relative abilities to induce a CTL response to the pathogenic antigens**

The cells are then sonicated, thus releasing phage that have successfully entered the target cells (thus protecting them from the polyclonal anti-M13 antiserum). These phage can, if desired, be amplified, experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis), and the selective cycle will be iteratively applied for, e.g., 3 - times. Individual phage from the final cycle can then be characterized with respect to their relative invasiveness. The best candidates can then be combined with gene III fusions that encode pathogenic epitopes of interest. These phage can be injected into mice and tested for their relative abilities to induce a CTL response to the pathogenic antigens.

Bacteriophage vaccine vehicles evolved for activity in mice according to the above methods will establish the principles for the evolution of similar vehicles for potent human vaccines. The ability to induce more rapid and potent CTL and neutralizing antibody responses with such vehicles is an important new tool for the evolution of improved countermeasures against pathogens of interest.

#### 2.6.5. EVOLUTION OF IMPROVED IMMUNOMODULATORY SEQUENCES

Cytokines can dramatically influence macrophage activation and  $T_H1/T_H2$  cell differentiation, and thereby the outcome of infectious diseases. In addition, recent studies strongly suggest that DNA itself can act as adjuvant by activating the cells of the immune system. Specifically, unmethylated CpG-rich DNA sequences were shown to enhance  $T_H1$  cell differentiation, activate cytokine synthesis by monocytes and induce proliferation of B lymphocytes. The invention thus provides methods for enhancing the immunomodulatory properties of genetic vaccines (a) by evolving the stimulatory properties of DNA itself and (b) by evolving genes encoding cytokines and related molecules that are involved in immune system regulation. These genes are then used in genetic vaccine vectors.

Of particular interest are IFN- $\gamma$  and IL-12, which skew immune responses towards a T helper I ( $T_H1$ ) cell phenotype and, thereby, improve the host's capacity to counteract pathogen invasions. Also provided are methods of obtaining improved immunomodulatory nucleic acids that are capable of inhibiting or enhancing activation, differentiation, or anergy of antigen-specific T cells. Because of the limited information about the structures and mechanisms that

regulate these events, molecular breeding C71 techniques of the invention provide much faster solutions than rational design.

5 The methods of the invention typically involve the use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other methods to create a library of experimentally generated (in vitro &/or in vivo) polynucleotides. The library is then screened to identify experimentally generated polynucleotides in the library, when included in a genetic vaccine vector or administered in conjunction with a genetic vaccine,  
10 are capable of enhancing or otherwise altering an immune response induced by the vector. The screening step, in some embodiments, can involve introducing a genetic vaccine vector that includes the experimentally generated polynucleotides into mammalian cells and determining whether the cells, or culture medium obtained by growing the cells, is capable of modulating an immune response.

15

Optimized recombinant vector modules obtained through polynucleotide reassembly (&/or one or more additional directed evolution methods described herein) are useful not only as components of genetic vaccine vectors, but also for production of polypeptides, e.g., modified cytokines and the like, that can be  
20 administered to a mammal to enhance or shift an immune response.

Polynucleotide sequences obtained using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention can be used as a component of a genetic vaccine, or can be used for production of cytokines and other immunomodulatory polypeptides  
25 that are themselves used as therapeutic or prophylactic reagents. If desired, the sequence of the optimized immunomodulatory polypeptide-encoding polynucleotides can be determined and the deduced amino acid sequence used to produce polypeptides using methods known to those of skill in the art.

### 2.6.5.1 IMMUNOSTIMULATORY DNA SEQUENCES

The invention provides methods of obtaining polynucleotides that are immunostimulatory when introduced into a mammal. Oligonucleotides that  
5 contain hexamers with a central CpG flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) efficiently induce cytokine synthesis and B cell proliferation (Krieg et al. (1995) Nature 374: 546; Klinman et al. (1996) Proc. Nat'l. Acad. Sci. USA 93: 2879; Pisetsky (1996) Immunity 5: 303-10) *in vitro* and act as adjuvants *in vivo*. Genetic vaccine vectors in which immunostimulatory  
10 sequence- (ISS) containing oligos are inserted have increased capacity to enhance antigen-specific antibody responses after DNA vaccination. The minimal length of an ISS oligonucleotide for functional activity *in vitro* is eight (Klinman et al., supra.). Twenty-mers with three CG motifs were found to be significantly more efficient in inducing cytokine synthesis than a 15- mer with two CG motifs (1d.).  
15 GGGG tetrads have been suggested to be involved in binding of DNA to cell surfaces (macrophages express receptors. for example scavenger receptors, that bind DNA) (Pisetsky et al., supra.).

According to the invention, a library is generated by subjecting to  
20 reassembly (&/or one or more additional directed evolution methods described herein) random DNA (e.g., fragments of human, murine, or other genomic DNA), oligonucleotides that contain known ISS, poly A, C, G or T sequences, or combinations thereof. The DNA, which includes at least first and second forms which differ from each other in two or more nucleotides, are reassembled (&/or  
25 subjected to one or more directed evolution methods described herein) to produce a library of experimentally generated polynucleotides.

The library is then screened to identify those experimentally generated polynucleotides that exhibit immunostimulatory properties, For example, the

library can be screened for induction cytokine production *in vitro* upon introduction of the library into an appropriate cell type. A diagram of this procedure is shown, described &/or referenced herein (including incorporated by reference). Among the cytokines that can be used as an indicator of

5 immunostimulatory activity are, for example, IL-2, IL-4, IL-5, IL- 6, IL- 10, IL- 12, IL- 13, IL- 15, and IFN- . One can also test for changes in ratios of IL-4/IFN- y, IL-4/IL-2, IL-5/ IFN- , IL-5/IL-2, IL- I 3/ IFN- , IL- 13/IL-2. An alternative screening method is the determination of the ability to induce proliferation of cells involved in immune responses, such as B cells, T cells, monocytes/macrophages,

10 total PBL, and the like. Other screens include detecting induction of APC activation based on changes in expression levels of surface antigens, such as B7-1 (CD80), B7-2 (CD86), MHC class I and II, and CD14.

Other useful screens include identifying, experimentally generated

15 polynucleotides that induce T cell proliferation. Because ISS sequences induce B cell activation, and because of several homologies between surface antigens expressed by T cells and B cells, polynucleotides can be obtained that have stimulatory activities on T cells.

20 Libraries of experimentally generated polynucleotides can also be screened for improved CTL and antibody responses *in vivo* and for improved protection from infection, cancer, allergy or autoimmunity. Experimentally generated polynucleotides that exhibit the desired property can be recovered from the cell and, if further improvement is desired, the reassembly (optionally in

25 combination with other directed evolution methods described herein) and screening, can be repeated. Optimized ISS sequences can used as an adjuvant separately from an actual vaccine, or the DNA sequence of interest can be fused to a genetic vaccine vector.



#### 2.6.5.2. CYTOKINES, CHEMOKINES, AND ACCESSORY MOLECULES

The invention also provides methods for obtaining optimized cytokines, cytokine antagonists, chemokines, and other accessory molecules that direct, inhibit, or enhance immune responses. For example, the methods of the invention can be used to obtain genetic vaccines and other reagents (e.g., optimized cytokines, and the like) that, when administered to a mammal, improve or alter an immune response. These optimized immunomodulators are useful for treating infectious diseases, as well as other conditions such as inflammatory disorders, in an antigen non-specific manner.

For example, the methods of the invention can be used to develop optimized immunomodulatory molecules for treating allergies. The optimized immunomodulatory molecules can be used alone or in conjunction with antigen-specific genetic vaccines to prevent or treat allergy. Four basic mechanisms are available by which one can achieve specific immunotherapy of allergy. First, one can administer a reagent that causes a decrease in allergen-specific  $T_H2$  cells. Second, a reagent can be administered that causes an increase in allergen-specific  $T_H1$  cells. Third, one can direct an increase in suppressive  $CD8^+$  T cells.

Finally, allergy can be treated by inducing anergy of allergen-specific T cells. In this Example, cytokines are optimized using the methods of the invention to obtain reagents that are effective in achieving one or more of these immunotherapeutic goals. The methods of the invention are used to obtain anti-allergic cytokines that have one or more properties such as improved specific activity, improved secretion after introduction into target cells, are effective at a lower dose than natural cytokines, and fewer side effects. Targets of particular interest include interferon- $\gamma$ , IL-10, IL-12, and antagonists of IL-4 and IL-13.

The optimized immunomodulators, or optimized experimentally generated polynucleotides that encode the immunomodulators, can be administered alone, or in combination with other accessory molecules. Inclusion of optimal concentrations of the appropriate molecules can enhance a desired immune response, and/or direct the induction or repression of a particular type of immune response. The polynucleotides that encode the optimized molecules can be included in a genetic vaccine vector, or the optimized molecules encoded by the genes can be administered as polypeptides.

In the methods of the invention, a library of experimentally generated polynucleotides that encode immunomodulators is created by subjecting substrate nucleic acids to a reassembly (&/or one or more additional directed evolution methods described herein) protocol, such as stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other method known to those of skill in the art. The substrate nucleic acids are typically two or more forms of a nucleic acid that encodes an immunomodulator of interest.

Cytokines are among the immunomodulators that can be improved using the 0 methods of the invention. Cytokine synthesis profiles play a crucial role in the capacity of the host to counteract viral, bacterial and parasitic infections, and cytokines can dramatically influence the efficacy of genetic vaccines and the outcome of infectious diseases. Several cytokines, for example IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, G-CSF, GM-CSF, IFN- $\alpha$ , IFN- $\beta$ , TGF- $\beta$ , TNF- $\alpha$ , TNF- $\beta$ , IL-20 (MDA-7), and flt-3 ligand have been shown stimulate immune responses *in vitro* or *in vivo*. Immune functions that can be enhanced using appropriate cytokines include, for example, B cell proliferation, Ig synthesis, Ig isotype switching, T cell proliferation and cytokine synthesis, differentiation of T<sub>H</sub>1 and T<sub>H</sub>2 cells,

activation and proliferation of CTLs, activation and cytokine production by monocytes/macrophages/ dendritic cells, and differentiation of dendritic cells from monocytes/macrophages.

- 5           In some embodiments, the invention provides methods of obtaining optimized immummodulators that can direct an immune response towards a  $T_H1$  or a  $T_H2$  response. The ability to influence the direction of immune responses in this manner is of great importance in development of genetic vaccines. Altering the type of  $T_H$  response can fundamentally change the outcome
- 10 of an infectious disease. A high frequency of  $T_H1$  cells generally protects from lethal infections with intracellular pathogens, whereas a dominant  $T_H2$  phenotype often results in disseminated, chronic infections. For example, in human, the  $T_H1$  phenotype is present in the tuberculoid (resistant) form of leprosy, while the  $T_H2$  phenotype is found in lepromatous, multibacillary (susceptible) lesions
- 15 (Yamamura et al. (1991) Science 254: 277). Late-stage AIDS patients have the  $T_H2$  phenotype. Studies in family members indicate that survival from meningococcal septicemia depends on the cytokine synthesis profile of PBL, with high IL-10 synthesis being associated with a high risk of lethal outcome and high TNF- being associated with a low risk. Similar examples are found in mice. For
- 20 example, BALB/c mice are susceptible to Leishmania major infection; these mice develop a disseminated fatal disease with a  $T_H2$  phenotype. Treatment with anti-IL-4 monoclonal antibodies or with IL-12 induces a  $T_H1$  response, resulting in healing. Anti-interferon- monoclonal antibodies exacerbate the disease. For some applications, it is preferable to direct an immune response in the direction of
- 25 a  $T_H2$  response.

For example, where increased mucosal immunity is desired, including protective immunity, enhancing the  $T_H2$  response can lead to increased antibody production, particularly IgA. T helper ( $T_H$ ) cells are probably the most important

regulators of the immune system. T<sub>H</sub> cells are divided into two subsets, based on their cytokine synthesis pattern (Mosmann and Coffman (1989) Adv. Immunol. 46: 111). T<sub>H</sub>1 cells produce high levels of the cytokines IL-2 and IFN- $\gamma$  and no or minimal levels of IL-4, IL-5 and IL-13. In contrast, T<sub>H</sub>2 cells produce high levels of IL-4, IL-5 and IL-13, and IFN- $\gamma$  production is minimal or absent. T<sub>H</sub>1 cells activate macrophages, dendritic cells and augment the cytolytic activity of CD8<sup>+</sup> cytotoxic T lymphocytes and natural killer (NK) cells (Paul and Seder (1994) Cell 76: 241), whereas T<sub>H</sub>2 cells provide efficient help for B cells and also mediate allergic responses due to the capacity of T<sub>H</sub>2 cells to induce IgE isotype switching and differentiation of B cells into IgE secreting cells (Punnonen et al. (1993) Proc. Nat'l. Acad. Sci. USA 90: 3730).

The screening methods for improved cytokines, chemokines, and other accessory molecules are generally based on identification of modified molecules that exhibit improved specific activity on target cells that are sensitive to the respective cytokine, chemokine, or other accessory molecules. A library of recombinant cytokine, chemokine, or accessory molecule nucleic acids can be expressed on phage or as purified protein and tested using *in vitro* cell culture assays, for example. Importantly, when analyzing the recombinant nucleic acids as components of DNA vaccines, one can identify the most optimal DNA sequences (in addition to the functions of the protein products) in terms of their immunostimulatory properties, transfection efficiency, and their capacity to improve the stabilities of the vectors. The identified optimized recombinant nucleic acids can then be subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

In one embodiment of the invention, cytokines are evolved that direct differentiation of T<sub>H</sub>1 cells. Because of their capacities to skew immune responses

towards a T<sub>H</sub>1 phenotype, the genes encoding interferon- (IFN- ) and interleukin-12 (IL-12) are preferred substrates for reassembly (&/or one or more additional directed evolution methods described herein) and selection in order to obtain maximal specific activity and capacity to act as adjuvants in genetic  
5 vaccinations. IFN- is a particularly preferred target for optimization using the methods of the invention because of its effects on the immune system, tumor cells growth and viral replication. Due to these activities, IFN- was the first cytokine to be used in clinical practice. Today, IFN- is used for a wide variety of applications, including several types of cancers and viral diseases. IFN- also  
10 efficiently directs differentiation of human T cells into T<sub>H</sub>1 phenotype (Parronchi et al. (1992) J Immunol. 149: 2977). However, it has not been thoroughly investigated in vaccination models, because, in contrast to human systems, it does not affect T<sub>H</sub>1 differentiation in mice.

15 The species difference was recently explained by data indicating that, like IL-12, IFN- induces STAT4 activation in human cells but not in murine cells, and STAT4 has been shown to be required in IL-12 mediated T<sub>H</sub>1 differentiation (Thierfelder et al. (1996) Nature 382: 171).

20 Family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is a preferred method for optimizing IFN- , using as substrates the mammalian IFN- . genes, which are 85% - 97% homologous. Greater 10<sup>26</sup> distinct recombinants can be generated from the natural diversity in these genes. To allow rapid parallel analysis of  
25 recombinant interferons, one can employ high throughput methods for their expression and biological assay as fusion proteins on bacteriophage.

Recombinants with improved potency and selectivity profiles are being selectively bred for improved activity. Variants which demonstrate improved

binding to IFN- receptors can be selected for further analysis using a screen for mutants with optimal capacity to direct T<sub>H</sub>1 differentiation. More specifically, the capacities of IFN- mutants to induce IL-2 and IFN-  $\gamma$  production in *in vitro* human T lymphocyte cultures can be studied by cytokine-specific ELISA and  
5 cytoplasmic cytokine staining and flow cytometry.

IL-12 is perhaps the most potent cytokine that directs T<sub>H</sub>1 responses, and it has also been shown to act as an adjuvant and enhance T<sub>H</sub>1 responses following genetic vaccinations (Kim et al. (1997) J Immunol. 158: 816). IL-12 is both  
10 structurally and functionally a unique cytokine. It is the only heterodimeric cytokine known to date, composed of a 35 kD light chain (p35) and a 40 kD heavy chain (p40) (Kobayashi et al (1989) J Exp. Med. 170: 827; Stem et al. (1990) Proc. Nat'l. Acad. Sci. USA 87: 6808).

15 Recently Lieschke et al. ((1997) Nature Biotech. 15: 35) demonstrated that a fusion between p35 and p40 genes results in a single gene that has activity comparable to that of the two genes expressed separately. These data indicate that it is possible to reassemble IL-12 genes as one entity, which is beneficial in designing the reassembly protocol (optionally in combination with other directed  
20 evolution methods described herein). Because of its T cell growth promoting activities, one can use normal human peripheral blood T cells in the selection of the most active IL-12 genes, enabling direct selection of IL-12 mutants with the most potent activities on human T cells. IL-12 mutants can be expressed in CHO cells, for example, and the ability of the supernatants to induce T cell proliferation  
25 determined. The concentrations of IL-12 in the supernatants can be normalized based on a specific ELISA that detects a tag fused to the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL- 12 molecules.

Incorporation of evolved IFN- and/or IL-12 genes into genetic vaccine vectors is expected to be safe. The safety of IFN- has been demonstrated in numerous clinical studies and in everyday hospital practice. A Phase II trial of IL-12 in the treatment of patients with renal cell cancer resulted in several  
5 unexpected adverse effects (Tahara et al. (1995) Human Gene Therapy 6: 1607). However, IL-12 gene as a component of genetic vaccines aims at high local expression levels, whereas the levels observed in circulation are minimal compared to those observed after systemic bolus injections. In addition, some of the adverse effects of systemic IL-12 treatments are likely to be related to its  
10 unusually long half-life (up to 48 hours in monkeys). stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly may allow selection for a shorter half- life, thereby reducing the toxicity even after high bolus doses.

15 In other cases, genetic vaccines that can induce  $T_H2$  responses are preferred, especially when improved antibody production is desired. As an example, IL-4 has been shown to direct differentiation of  $T_H2$  cells (which produce high levels of IL-4, IL-5 and IL-13, and mediate allergic immune responses). Immune responses that are skewed towards  $T_H2$  phenotype are  
20 preferred when genetic vaccines are used to immunize against autoimmune diseases prophylactically.  $T_H1$  responses are also preferred when the vaccines are used to treat and modulate existing autoimmune responses, because autoreactive T cells are generally of  $T_H1$  phenotype (Liblau et al. (1995) Immunol. Today 16:34- 38). IL-4 is also the most potent cytokine in induction of IgE synthesis; IL-  
25 4 deficient mice are unable to produce IgE. Asthma and allergies are associated with an increased frequency of IL-4 producing cells, and are genetically linked to the locus encoding IL-4, which is on chromosome 5 (in close proximity to genes encoding IL-3, IL-5, IL-9, IL-13 and GM-CSF). IL-4, which is produced by activated T cells, basophils and mast cells, is a protein that has 153 amino acids

and two potential N-glycosylation sites. Human IL-4 is only approximately 50% identical to mouse IL-4, and IL-4 activity is species-specific. In human, IL-13 has activities similar to those of IL-4, but IL-13 is less potent than IL-4 in inducing IgE synthesis. IL-4 is the only cytokine known to direct T<sub>H</sub>2 differentiation.

5

Improved IL-2 agonists are also useful in directing T<sub>H</sub>2 cell differentiation, whereas improved IL-4 antagonists can direct T<sub>H</sub>1 cell differentiation. Improved IL-4 agonists and antagonists can be generated by the reassembly (optionally in combination with other directed evolution methods  
10 described herein) of IL-4 or soluble IL-4 receptor. The IL-4 receptor consists of an IL-4R  $\alpha$ -chain (140 kD high-affinity binding unit) and an IL-2R  $\gamma$ -chain (these cytokine receptors share a common  $\gamma$ -chain). The IL-4R  $\alpha$ -chain is shared by IL-4 and IL-13 receptor complexes. Both IL-4 and IL-13 induce phosphorylation of the IL-4R  $\gamma$ -chain, but expression of IL-4R  $\alpha$ -chain alone on  
15 transfectants is not sufficient to provide a functional IL-4R. Soluble IL-4 receptor currently in clinical trials for the treatment of allergies. Using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention, one can evolve a soluble IL-4 receptor that has improved affinity for IL-4. Such receptors are useful for the  
20 treatment of asthma and other T<sub>H</sub>2 cell mediated diseases, such as severe allergies. The reassembly (optionally in combination with other directed evolution methods described herein) reactions can take advantage of natural diversity present in cDNA libraries from activated T cells from human and other primates. In a typical embodiment, a experimentally evolved (e.g. by polynucleotide  
25 reassembly &/or polynucleotide site-saturation mutagenesis) IL-4R  $\alpha$ -chain in library is expressed on a phage, and mutants that bind to IL-4 with improved affinity are identified. The biological activity of the selected mutants is then assayed using cell-based assays.



IL-2 and IL-15 are also of particular interest for use in genetic vaccines. IL-2 acts as a growth factor for activated B and T cells, and it also modulates the functions of NK-cells. IL-2 is predominantly produced by T<sub>H</sub>1-like T cell clones, and, therefore, it is considered mainly to function in delayed type hypersensitivity reactions. However, IL-2 also has potent, direct effects on proliferation and Ig-synthesis by B cells. The complex immunoregulatory properties of IL-2 are reflected in the phenotype of IL-2 deficient mice, which have high mortality at young age and multiple defects in their immune functions including spontaneous development of inflammatory bowel disease. IL-15 is a more recently identified cytokine produced by multiple cell types. IL-15 shares several, but not all, activities with IL-2. Both IL-2 and IL-15 induce B cell growth and differentiation. However, assuming that IL-15 production in IL-2 deficient mice is normal, it is clear that IL-15 cannot substitute for the function of IL-2 *in vivo*, since these mice have multiple immunodeficiencies. IL-2 has been shown to synergistically enhance IL-10- induced human Ig production in the presence of anti-CD40 mAbs, but it antagonized the effects of IL-4. IL-2 also enhances IL-4-dependent IgE synthesis by purified B cells. On the other hand, IL-2 was shown to inhibit IL-4-dependent murine IgG1 and IgE synthesis both *in vitro* and *in vivo*. Similarly, IL-2 inhibited IL-4-dependent human IgE synthesis by unfractionated human PBMC, but the effects were less significant than those of IFN- $\gamma$  or IFN- $\alpha$ . Due to their capacities to activate both B and T cells, IL-2 and IL-15 are useful in vaccinations. In fact, IL-2, as protein and as a component of genetic vaccines, has been shown to improve the efficacy of the vaccinations. Improving the specific activity and/or expression levels/kinetics of IL-2 and IL-15 through use of the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention increases the advantageous effects compared to wild-type IL-2 and IL-15.

Another cytokine of particular interest for optimization and use in genetic vaccines according to the methods of the invention is interleukin-6. IL-6 is a monocyte- derived cytokine that was originally described as a B cell differentiation factor or B cell stimulatory factor-2 because of its ability to  
5 enhance Ig levels secreted by activated B cells.

IL-6 has also been shown to enhance IL-4-induced I-E synthesis. It has also been suggested that IL-6 is an obligatory factor for human IgE synthesis, because neutralizing anti-IL-6 mAbs completely blocked IL-4-induced IgE  
10 synthesis. IL-6 deficient mice have impaired capacity to produce IgA. Because of its potent activities on the differentiation of B cells, IL- 6 can enhance the levels of specific antibodies produced following vaccination. It is particularly useful as a component of DNA vaccines because high local concentrations can be achieved, thereby providing the most potent effects on the cells adjacent to the transfected  
15 cells expressing the immunogenic antigen. IL-6 with improved specific activity and/or with improved expression levels, obtained by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, will have more beneficial effects than the wild-type IL-6.

20

Interleukin-8 is another example of a cytokine that, when modified according to the methods of the invention, is useful in genetic vaccines. IL-8 was originally identified as a monocyte-derived neutrophil chemotactic and activating factor. Subsequently, IL-8 was also shown to be chemotactic for T cells and to  
25 activate basophils resulting in enhanced histamine and leukotriene release from these cells. Furthermore, IL-8 inhibits adhesion of neutrophils to cytokine-activated endothelial cell monolayers, and it protects these cells from neutrophil-mediated damage. Therefore, endothelial cell derived IL-8 was suggested to 3 \_31 attenuate inflammatory events occurring in the proximity of blood vessel walls.

IL-8 also modulates immunoglobulin production, and inhibits IL-4-induced IgG4 and IgE synthesis by both unfractionated human PBMC and purified B cells *in vitro*. This inhibitory effect was independent of IFN- $\gamma$ , IFN- $\alpha$  or prostaglandin E2. In addition, IL-8 inhibited spontaneous IgE synthesis by PBMC derived from  
5 atopic patients. Due to its capacity to attract inflammatory cells, IL-8, like other chemotactic agents, is useful in potentiating the functional properties of vaccines, including DNA vaccines (acting as an adjuvant). The beneficial effects of IL-8 can be improved by using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of  
10 the invention to obtain IL-8 with improved specific activity and/or with improved expression in target cells.

Interleukin-5, and antagonists thereof, can also be optimized using the methods of the invention for use in genetic vaccines. IL-5 is primarily produced  
15 by T<sub>H</sub>2-type T cells and appears to play an important role in the pathogenesis of allergic disorders because of its ability to induce eosinophilia. IL-5 acts as an eosinophil differentiation and survival factor in both mouse and man. Blocking IL-5 activity by use of neutralizing monoclonal antibodies strongly inhibits pulmonary eosinophilia and hyperactivity in mouse models, and IL-5 deficient  
20 mice do not develop eosinophilia. These data also suggest that IL-5 antagonists may have therapeutic potential in the treatment of allergic eosinophilia.

IL-5 has also been shown to enhance both proliferation of, and Ig synthesis by, activated mouse and human B cells. However, other studies  
25 suggested that IL-5 has no effect on proliferation of human B cells, whereas it activated eosinophils. IL-5 apparently is not crucial for maturation or differentiation of conventional B cells, because antibody responses in IL-5 deficient mice are normal. However, these mice have a developmental defect in their CD5<sup>+</sup> B cells indicating that IL-5 is required for normal differentiation of

this B cell subset in mice. At suboptimal concentrations of IL-4, IL-5 was shown to enhance IgE synthesis by human B cells *in vitro*. Furthermore, a recent study suggested that the effects of IL-5 on human B cells depend on the mode of B cell stimulation. IL-5 significantly enhanced IgM synthesis by B cells stimulated with  
5 *Moraxella catarrhalis*. In addition, IL-5 synergized with suboptimal concentrations of IL-2, but had no effect on I- synthesis by SAC-activated B cells. Activated human B cells also expressed IL-5 mRNA suggesting that IL-5 may also regulate B cell function, including I-E synthesis, by autocrine mechanisms.

10 The invention provides methods of evolving an IL-5 antagonist that efficiently binds to and neutralizes IL-5 or its receptor. These antagonists are useful as a component of vaccines used for prophylaxis and treatment of allergies. Nucleic acids encoding IL-5, for example, from human and other mammalian species, are experimentally evolved (e.g. by polynucleotide reassembly &/or  
15 polynucleotide site-saturation mutagenesis) and screened for binding to immobilized IL-5R for the initial screening. Polypeptides that exhibit the desired effect in the initial screening assays can then be screened for the highest biological activity using assays such as inhibition of growth of IL-5 dependent cells lines cultured in the presence of recombinant wild-type IL-5. Alternatively,  
20 experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL-5R - chains are screened for improved binding to IL-5.

Tumor necrosis factors ( and ) and their receptors are also suitable  
25 targets for modification and use in genetic vaccines. TNF- , which was originally described as cachectin because of its ability to cause necrosis of tumors, is a 17 kDa protein that is produced in low quantities by almost all cells in the human body following activation. TNF- acts as an endogenous pyrogen and induces the synthesis of several proinflammatory cytokines, stimulates the production of acute

phase proteins, and induces proliferation of fibroblasts. TNF- $\alpha$  plays a major role in the pathogenesis of endotoxin shock. A membrane-bound form of TNF- $\alpha$  (mTNF- $\alpha$ ), which is involved in interactions between B- and T-cells, is rapidly upregulated within four hours of T cell activation. mTNF- $\alpha$  plays a role in the polyclonal B cell activation observed in patients infected with HIV. Monoclonal antibodies specific for mTNF- $\alpha$  or the p55 TNF- $\alpha$  receptor strongly inhibit IgE synthesis induced by activated CD4<sup>+</sup> T cell clones or their membranes. Mice deficient for p55 TNF- $\alpha$  R are resistant to endotoxic shock, and soluble TNF- $\alpha$  R prevents autoimmune diabetes mellitus in NOD mice. Phase III trials using sTNF- $\alpha$  R in the treatment of rheumatoid arthritis are in progress, after promising results obtained in the phase II trials.

The methods of the invention can be used to, for example, evolve a soluble TNF- $\alpha$  R that has improved affinity, and thus is capable of acting as an antagonist for TNF activity. Nucleic acids that encode TNF- $\alpha$  R and exhibit sequence diversity, such as the natural diversity observed in cDNA libraries from activated T cells of human and other primates, are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis). The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids are expressed, e.g., on phage, after which mutants are selected that bind to TNF- $\alpha$  with improved affinity. If desired, the improved mutants can be subjected to further assays using biological activity, and the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes can be subjected to one or more rounds of reassembly (optionally in combination with other directed evolution methods described herein) and screening.

Another target of interest for application of the methods of the invention is interferon- $\gamma$ , and the evolution of antagonists of this cytokine. The receptor for

IFN-  $\gamma$  consists of a binding component glycoprotein of 90 kD, a 228 amino acid extracellular portion, a transmembrane region, and a 222 amino acid intracellular region. Glycosylation is not required for functional activity. A single chain provides high affinity binding ( $10^{-9}$ - $10^{-10}$  M), but is not sufficient for signaling.

5 Receptor components dimerize upon ligand binding.

The mouse IFN-  $\gamma$  receptor is 53% identical to that of mouse at the amino acid level. The human and mouse receptors only bind human and mouse IFN-  $\gamma$ , respectively. Vaccinia, cowpox and camelpox viruses have homologues of sIFN-  
10 R, which have relatively low amino acid sequence similarity (~20%), but are capable of efficient neutralization of IFN-  $\gamma$  *in vitro*. These homologues bind human, bovine, rat (but not mouse) IFN-  $\gamma$ , and may have *in vivo* activity as IFN-  $\gamma$  antagonists. All eight cysteines are conserved in human, mouse, myxoma and Shope fibroma virus (6 in vaccinia virus) IFN-  $\gamma$  R polypeptides, indicating  
15 similar 3-D structures. An extracellular portion of m IFN-  $\gamma$  R with a kD of 100-300 pM has been expressed in insect cells. Treatment of NZB/W mice (a mouse model of human SLE) with msIFN-  $\gamma$  receptor (100 mg/three times a week i.p.) inhibits the onset of glomerulonephritis. All mice treated with sIFN-  $\gamma$  or anti-IFN-  $\gamma$  niAbs were alive 4 weeks after the treatment was discontinued, compared  
20 with 50% in a placebo group, and 78% of IFN-  $\gamma$  -treated mice died.

The methods of the invention can be used to evolve soluble IFN-  $\gamma$  R receptor polypeptides with improved affinity, and to evolve IFN-  $\gamma$  with improved specific activity and improved capacity to activate cellular immune responses. In  
25 each case nucleic acids encoding the respective polypeptide, and which exhibit sequence diversity (e.g., that observed in cDNA libraries from activated T cells from human and other primates), are subjected to reassembly (&/or one or more additional directed evolution methods described herein) and screened to identify those recombinant nucleic acids that encode a polypeptide having improved

activity. In the case of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IFN- R , the library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids can be expressed on phage, which are  
5 screened to identify mutants that bind to IFN- with improved affinity. In the case of IFN- , the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library is analyzed for improved specific activity and improved activation of the immune system, for example, by using activation of monocytes/macrophages as an assay. The evolved IFN-  
10 molecules can improve the efficacy of vaccinations (e.g. when used as adjuvants). Diseases that can be treated using high-affinity sIFN- R polypeptides obtained using the methods of the invention include, for example, multiple sclerosis, systemic lupus erythematosus (SLE), organ rejection after treatment, and graft versus host disease. Multiple sclerosis, for example, is characterized by increased  
15 expression of IFN- in the brain of the patients, and increased production of IFN- by patients' T cells *in vitro*. IFN- treatment has been shown to significantly exacerbate the disease (in contrast to EAE in mice).

Transforming growth factor (TGF)- is another cytokine that can be  
20 optimized for use in genetic vaccines using the methods of the invention. TGF- has growth regulatory activities on essentially all cell types, and it has also been shown to have complex modulatory effects on the cells of the immune system. TGF- inhibits proliferation of both B and T cells, and it also suppresses development of and differentiation of cytotoxic T cells and NK cells, TGF- has  
25 been shown to direct IgA switching in both murine and human B cells. It was also shown to induce germline a transcription in murine and human B cells, supporting the conclusion that TGF- can specifically induce IgA switching.

Due to its capacity to direct IgA switching, TGF- $\beta$  is useful as a component of DNA vaccines which aim at inducing potent mucosal immunity, *e.g.* vaccines for diarrhea. Also, because of its potent anti-proliferative effects TGF- $\beta$  is useful as a component of therapeutical cancer vaccines. TGF- $\beta$  with improved specific activity and/or with improved expression levels/kinetics will have increased beneficial effects compared to the wild-type TGF- $\beta$ .

Cytokines that can be optimized using the methods of the invention also include granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF). These cytokines induce differentiation of bone marrow stem cell into granulocytes/macrophages. Administration of G-CSF and GM-CSF significantly improve recovery from bone marrow (BM) transplantation and radiotherapy, reducing infections and time the patients have to spend in hospitals. GM-CSF enhances antibody production following DNA vaccination. G-CSF is a 175 amino acid protein, while GM-CSF has 127 amino acids. Human G-CSF is 73% identical at the amino acid level to murine G-CSF and the two proteins show species cross-reactivity. G-CSF has a homodimeric receptor (dimeric with kD of  $\sim 200$  pM, monomeric  $\sim 2.4$  nM), and the receptor for GM-CSF is a three subunit complex. Cell lines transfected with cDNA encoding G-CSF R proliferate in response to G-CSF. Cell lines dependent of GM-CSF available (such as TF-1). G-CSF is nontoxic and is presently working very well as a drug. However, the treatment is expensive, and more potent G-CSF might reduce the cost for patients and to the health care. Treatments with these cytokines are typically short-lasting and the patients are likely to never need the same treatment again reducing likelihood of problems with immunogenicity.

The methods of the invention are useful for evolving G-CSF and/or GM-CSF which have improved specific activity, as well as other polypeptides that have G-CSF and/or GM-CSF activity. G-CSF and/or GM-CSF nucleic acids



having sequence diversity, e.g., those obtained from cDNA libraries from diverse species, are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) to create a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) G-CSF and/or GM-CSF genes. These libraries can be screened by, for example, picking colonies, transfecting the plasmids into a suitable host cell (e.g., CHO cells), and assaying the supernatants using receptor-positive cell lines. Alternatively, phage display or related techniques can be used, again using receptor-positive cell lines. Yet another screening method involves transfecting the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes into G-CSF/GM-CSF-dependent cell lines. The cells are grown one cell per well and/or at very low density in large flasks, and the cells that grow fastest are selected. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes from these cells are isolated; if desired, these genes can be used for additional rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

Ciliary neurotrophic factor (CNTF) is another suitable target for application of the methods of the invention. CNTF has 200 amino acids which exhibit 80% sequence identity between rat and rabbit CNTF polypeptides. CNTF has IL-6-like inflammatory effects, and induces synthesis of acute phase proteins. CNTF is a cytosolic protein which belongs to the IL-6/IL-11/LIF/oncostatin M - family, and becomes biologically active only after becoming available either by cellular lesion or by an unknown release mechanism. CNTF is expressed by myelinating Schwann cells, astrocytes and sciatic nerves.

Structurally, CNTF is a dimeric protein, with a novel anti-parallel arrangement of the subunits. Each subunit adopts a double crossover four-helix

bundle fold, in which two helices contribute to the dimer interface. Lys-155 mutants lose activity, and some Glu-153 mutants have 5-10 higher biological activity. The receptor for CNTF consists of a specific CNTF receptor chain, gp130, and a LIF- receptor. The CNTFR -chain lacks a transmembrane domain portion, instead being GPI-anchored. At high concentration, CNTF can mediate CNTFR-independent responses. Soluble CNTFR binds CNTF and thereafter can bind to LIFR and induce signaling through gp 130. CNTF enhances survival of several types of neurons, and protects neurons in an animal model of Huntington disease (in contrast to NGF, neurotrophic factor, and neurotrophin-3).

5 CNTF receptor knockout mice have severe motor neuron deficits at birth, and CNTF knockout mice exhibit such deficits postnatally. CNTF also reduces obesity in mouse models. Decreased expression of CNTF is sometimes observed in psychiatric patients. Phase I studies in patients with ALS (annual incidence ~1/100 000, 5% familial cases, 90% die within 6 years) found significant side

10 effects after doses higher than 5 mg/kg/day subcutaneously (including anorexia, weight loss, reactivation of herpes simplex virus (HSV1), cough, increased oral secretions). Antibodies against CNTF were detected in almost all patients, thus illustrating the need for alternative CNTF with different immunological properties.

20

The reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention can be used to obtain modified CNTF polypeptides that exhibit decreased immunogenicity *in vivo*; higher also obtainable using the methods. reassembly (optionally in combination

25 with other directed evolution methods described herein) is conducted using nucleic acids encoding CNTF. In a preferred embodiment, an IL-6/LIF/(CNTF) hybrid is obtained by reassembly (optionally in combination with other directed evolution methods described herein) using an excess of oligonucleotides that

encode to the receptor binding sites of CNTF. Phage display can then be used to test for lack of binding to the IL-6/LIF receptor.

5 This initial screen is followed by a test for high affinity binding to the CNTF receptor, and, if desired, functional assays using CNTF responsive cell lines. The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) CNTF polypeptides can be tested to identify those that exhibit reduced immunogenicity upon administration to a mammal.

10

Another way in which the reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention can be used to optimize CNTF is to improve secretion of the polypeptide. When a CNTF cDNA is operably linked to a leader sequence of  
15 hNGF, only 35-40 percent of the total CNTF produced is secreted.

Target diseases for treatment with optimized CNTF, using either the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) gene in an expression vector as in DNA vaccines, or  
20 a purified protein, include obesity, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), diabetic neuropathy, stroke, and brain surgery.

Polynucleotides that encode chemokines can also be optimized using the methods of the invention and included in a genetic vaccine vector. At least three  
25 classes of chemokines are known, based on structure: C chemokines (such as lymphotactin), C-C chemokines (such as MCP-1, MCP-2, MCP-3, MCP-4, MIP-1a, MIP-1b, RANTES), C-X-C chemokines (such as IL-8, SDF-1, ELR, Mig, IP 10) (Premack and Schall (1996) Nature Med. 2: 1174). Chemokines can attract other cells that mediate immune and inflammatory functions, thereby potentiating

the immune response. Cells that are attracted by different types of chemokines include, for example, lymphocytes, monocytes and neutrophils. Generally, C-X-C chemokines are chemoattractants for neutrophils but not for monocytes, C-C chemokines attract monocytes and lymphocytes but not neutrophils, C chemokine  
5 attracts lymphocytes.

Genetic vaccine vectors can also include optimized experimentally generated polynucleotides that encode surface-bound accessory molecules, such as those that are involved in modulation and potentiation of immune responses.  
10 These molecules, which include, for example, B7-1 (CD80), B7-2 (CD86), CD40, ligand for CD40, CTLA-4, CD28, and CD 150 (SLAM), can be subjected to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to obtain variants have altered and/or improved activities.

15

Optimized experimentally generated polynucleotides that encode CD1 molecules are also useful in a genetic vaccine vector for certain applications. CD1 are nonpolymorphic molecules that are structurally and functionally related to MEC molecules. Importantly, CD I has MHC-like activities, and it can function as  
20 an antigen presenting molecule (Porcelli (1995) Adv. Immunol. 59: 1). CD1 is highly expressed on dendritic cells, which are very efficient antigen presenting cells. Simultaneous transfection of target cells with DNA vaccine vectors encoding CD1 and an antigen of interest is likely to boost the immune response. Because CD1 cells, in contrast to MHC molecules, exhibit limited allelic diversity  
25 in an outbred population (Porcelli, supra.), large populations of individuals with different genetic backgrounds can be vaccinated with one CD1 allele. The functional properties of CD1 molecules can be improved by the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention.

Optimized recombinant TAP genes and/or gene products can also be included in a genetic vaccine vector. TAP genes and their optimization for various purposes are discussed in more detail below. Moreover, heat shock proteins (HSP), such as HSP70, can also be evolved for improved presentation and processing of antigens. HSP70 has been shown to act as adjuvant for induction of CD8<sup>+</sup> T cell activation and it enhances immunogenicity of specific antigenic peptides (Blachere et al. (1997) J Exp. Med. 186:1315-22). When HSP70 is encoded by a genetic vaccine vector, it is likely to enhance presentation and processing of antigenic peptides and thereby improve the efficacy of the genetic vaccines. stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be used to further improve the properties, including adjuvant activity, of heat shock proteins, such as HSP70.

Recombinantly produced cytokine, chemokine, and accessory molecule polypeptides, as well as antagonists of these molecules, can be used to influence the type of immune response to a given stimulus. However, the administration of polypeptides sometimes has shortcomings, including short half life, high expense, difficult to store (must be stored at 4°C), and a requirement for large volumes. Also, bolus injections can sometimes cause side effects. Administration of polynucleotides that encode the recombinant cytokines or other molecules overcomes most or all of these problems. DNA, for example, can be prepared in high purity, is stable, temperature resistant, noninfectious, easy to manufacture. In addition, polynucleotide-mediated administration of cytokines can provide long-lasting, consistent expression, and administration of polynucleotides in general is regarded as being safe.

The functions of cytokines, chemokines and accessory molecules are redundant and pleiotropic, and therefore can be difficult to determine which

cytokines or cytokine combinations are the most potent in inducing and enhancing antigen specific immune responses following vaccination. Furthermore, the most useful combination of cytokines and accessory molecules is typically different depending on the type of immune C, response that is desired following

5 vaccination. As an example, IL-4 has been shown to direct differentiation of  $T_H2$  cells (which produce high levels of IL-4, IL-5 and IL-13, and mediate allergic immune responses), whereas IFN- and IL-12 direct differentiation of  $T_H1$  cells (which produce high levels of IL-2 and IFN- ), and mediate delayed type

10 accessory molecules is also likely to depend on the antigen used in the vaccination. The invention provides a solution to this problem of obtaining an optimized genetic vaccine cocktail. Different combinations of cytokines, chemokines and accessory molecules are assembled into vectors using the methods described herein. These vectors are then screened for their capacity to

15 induce immune responses *in vivo* and *in vitro*.

Large libraries of vectors, generated by polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein) and combinatorial

20 molecular biology, are screened for maximal capacity to direct immune responses towards, for example, a  $T_H1$  or  $T_H2$  phenotype, as desired. A library of different vectors can be generated by assembling different evolved promoters, (evolved) cytokines, (evolved) cytokine antagonists, (evolved) chemokines, (evolved) accessory molecules and immunostimulatory sequences, each of which can be

25 prepared using methods described herein. DNA sequences and compounds that facilitate the transfection and expression can be included. If the pathogen(s) is known, specific DNA sequences encoding immunogenic antigens from the pathogen can be incorporated into these vectors providing protective immunity against the pathogen(s) (as in genetic vaccines).

Initial screening is preferably carried out *in vitro*. For example, the library can be introduced into cells which are tested for ability to induce differentiation of T cells capable of producing cytokines that are indicative of the type of immune response desired. For a T<sub>H</sub>1 response, for example, the library is screened to identify experimentally generated polynucleotides that are capable of inducing T cells to produce IL-2 and IFN- $\gamma$ , while screening for induction of T cell production of IL-4, IL-5, and IL-13 is performed to identify experimentally generated polynucleotides that favor a T<sub>H</sub>2 response.

10

Screening can also be conducted *in vivo*, using animal models. For example, vectors produced using the methods of the invention can be tested for ability to protect against a lethal infection. Another screening method involves injection of *Leishmania major* parasites into footpads of BALB/c mice (nonhealer). Pools of plasmids are injected i.v., i.p. or into footpads of these mice and the size of the footpad swelling is followed. Yet another *in vivo* screening method involves detection of IgE levels after infection with *Nippostrongylus brasiliensis*. High levels indicate a T<sub>H</sub>2 response, while low levels of IgE indicate a T<sub>H</sub>1 response.

20

Successful results in animal models are easy to verify in humans. *In vitro* screening can be conducted to test for human T<sub>H</sub>1 or T<sub>H</sub>2 phenotype, or for other desired immune response. Vectors can also be tested for ability to induce protection against infection in humans. Because the principles of immune functions are similar in a wide variety of infections, immunostimulating DNA vaccine vectors may not only be useful in the treatment of a number of infectious diseases but also in prevention of the infections, when the vectors are delivered to the sites of the entry of the pathogen (e.g., the lung or gut).

25

### 2.6.5.3. AGONISTS OR ANTAGONISTS OF CELLULAR RECEPTORS

The invention also provides methods for obtaining optimized  
5 experimentally generated polynucleotides that encode a peptide or polypeptide  
that can interact with a cellular receptor that is involved in mediating an immune  
response. The optimized experimentally generated polynucleotides can act as an  
agonist or an antagonist of the receptor.

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#### Cytokine antagonists can be used as components of genetic vaccine cocktails

Blocking immunosuppressive cytokines, rather than adding single  
proinflammatory cytokines, is likely to potentiate the immune response in a more  
15 general manner, because several pathways are potentiated at the same time. By  
appropriate choice of antagonist, one can tailor the immune response induced by a  
genetic vaccine in order to obtain the response that is most effective in achieving  
the desired effect. Antagonists against any cytokine can be used as appropriate;  
particular cytokines of interest for blocking include, for example, IL-4, IL-13, IL-  
20 10, and the like.

The invention provides methods of obtaining cytokine antagonists that  
exhibit greater effectiveness in blocking the action of the respective cytokine.  
Polynucleotides that encode improved cytokine antagonists can be obtained by  
25 using polynucleotide (e.g. gene, promoter, enhancer, intron, & the like)  
reassembly (optionally in combination with other directed evolution methods  
described herein) to generate a recombinant library of polynucleotides which are  
then screened to identify those that encode an improved antagonist. As substrates  
for the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-



stochastic polynucleotide reassembly, one can use, for example, polynucleotides that encode receptors for the respective cytokine. At least two forms of the substrate will be present in the reassembly (&/or one or more additional directed evolution methods described herein) reaction, with each form differing from the other in at least one nucleotide position. In a preferred embodiment, the different forms of the polynucleotide are homologous cytokine receptor genes from different organisms. The resulting library of experimentally generated polynucleotides is then screened to identify those that encode cytokine antagonists with the desired affinity and biological activity.

10

As one example of the type of effect that one can achieve by including a cytokine antagonist in a genetic vaccine cocktail, as well as how the effect can be improved using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention, IL-10 is discussed. The same rationale can be applied to obtaining and using antagonists of other cytokines. Interleukin-10 (IL-10) is perhaps the most potent anti-inflammatory cytokine known to date. IL-10 inhibits a number of pathways that potentiate inflammatory responses. The biological activities of IL-10 include inhibition of MHC class II expression on monocytes, inhibition of production of IL-1, IL-6, IL-12, TNF- $\alpha$  by monocytes/macrophages, and inhibition of proliferation and IL-2 production by T lymphocytes. The significance of IL-10 as a regulatory molecule of immune and inflammatory responses was clearly demonstrated in IL-10 deficient mice.

20

These mice are growth-retarded, anemic and spontaneously develop an inflammatory bowel disease (Kuhn et al. (1993) Cell 75: 263). In addition, both innate and acquired immunity to *Listeria monocytogenes* were shown to be elevated in IL-10 deficient mice (Dai et al. (1997) J Immunol. 158: 2259). It has also been suggested that genetic differences in the levels of IL-10 production may

25

affect the risk of patients to die from complications meningococcal. infection. Families with high IL-10 production had 20-fold increased risk of fatal outcome of meningococcal. disease (Westendorp et al. (1997) Lancet 349: 170).

5 IL- 10 has been shown to activate normal and malignant B cells *in vitro*, but it does not appear to be a major growth promoting cytokine for normal B cells *in vivo*, because IL-10 deficient mice have normal levels of B lymphocytes and Ig in their circulation. In fact, there is evidence that IL-10 can indirectly  
10 downregulate B cell function through inhibition of the accessory cell function of monocytes. However, IL-10 appears to play a role in the growth and expansion of malignant B cells. Anti-IL-10 monoclonal antibodies and IL- 10 antisense oligonucleotides have been shown to inhibit transformation of B cells by EBV *in vitro*. In addition, B cell lymphomas are associated with EBV and most EBV<sup>+</sup> lymphomas produce high levels of IL-10, which is derived both from the human  
15 gene and the homologue of IL-10 encoded by EBV. AIDS-related B cell lymphomas also secrete high levels of IL-10. Furthermore, patients with detectable serum IL-10 at the time of diagnosis of intermediate/high-grade non-Hodgkin's lymphoma have short survival, further suggesting IDID In a role for IL- 10 in the pathogenesis of B cell malignancies.

20

Antagonizing IL- 10 *in vivo* can be beneficial in several infectious and malignant diseases, and in vaccination. The effect of blocking of IL-10 is an enhancement of immune responses that is independent of the specificity of the response. This is useful in vaccinations and in the treatment of serious infectious  
25 diseases. Moreover, an IL-10 antagonist is useful in the treatment of B cell malignancies which exhibit overproduction of IL-10 and viral IL-10, and it may also be useful in boosting general anti-tumor immune response in cancer patients. Combining an IL-10 antagonist with gene therapy vectors may be useful in gene therapy of tumor cells in order to obtain maximal immune response against the

tumor cells. If the reassembly (optionally in combination with other directed evolution methods described herein) of IL-10 results in IL-10 with improved specific activity, this IL-10 molecule would have potential in the treatment of autoimmune diseases and inflammatory bowel diseases. IL-10 with improved  
5 specific activity may also be useful as a component of gene therapy vectors in reducing the immune response against vectors which are recognized by memory cells and it may also reduce the immunogenicity of these vectors.

An antagonist of IL-10 has been made by generating a soluble form of IL-  
10 10 receptor (sIL-10R; Tan et al. (1995) J Biol. Chem. 270: 12906). However, sIL-10R binds IL-10 with Kd of 560 pM, whereas the wild-type, surface-bound receptor has affinity of 35- 200 pM. Consequently, 150-fold molar excess of sIL-10R is required for half-maximal inhibition of biological function of IL-10. Moreover, affinity of viral IL-10 (IL-10 homologue encoded by Epstein-Barr  
15 virus) to sIL-10R is more than 1000 fold less than that of hIL-10, and in some situations, such as when treating EBV-associated B cell malignancies, it may be beneficial if one can also block the function of viral IL-10. Taken together, this soluble form of IL-10R is unlikely to be effective in antagonizing IL-10 *in vivo*.

20 To obtain an IL-10 antagonist that has sufficient affinity and antagonistic activity to function *in vivo*, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be performed using polynucleotides that encode IL-10 receptor. IL-10 receptor with higher than normal affinity will function as an IL- 10 antagonist, because it strongly reduces  
25 the amount of IL-10 available for binding to functional, wild-type IL-10R. In a preferred embodiment, IL-10R is experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) using homologous cDNAs encoding IL-10R derived from human and other mammalian species.

An alignment of human and mouse IL-10 receptor sequences is shown, described &/or referenced herein (including incorporated by reference) to illustrate the feasibility of family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly when evolving IL-10 receptors with improved affinity. A phage library of IL-10 receptor recombinants can be screened for improved binding of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL-10R to human or viral IL-10. Wild- type IL-10 and/or viral IL- 0 are added at increasing concentrations to demand for higher affinity. Phage bound to IL-10 can be recovered using anti-IL-10 monoclonal antibodies. If desired, the shuffling can be repeated one or more times, after which the evolved soluble IL- 10R is analyzed in functional assays for its capacity to neutralize the biological activities of IL- 10/viral IL- 10. More specifically, evolved soluble IL-10R is studied for its capacity to block the inhibitory effects of IL-10 on cytokine synthesis and MHC class II expression by monocytes, proliferation by T cells, and for its capacity to inhibit the enhancing effects of IL-10 on proliferation of B cells activated by anti-CD40 monoclonal antibodies.

An IL-10 antagonist can also be generated by evolving IL-10 to obtain variants that bind to IL-10R with higher than wild-type affinity, but without receptor activation. The advantage of this approach is that one can evolve an IL- 10 molecule with improved specific activity using the same methods. In a preferred embodiment, IL-10 is experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) using homologous cDNAs encoding IL-10 derived from human and other mammalian species. In addition, a gene encoding viral IL-10 can be included in the reassembly (optionally in combination with other directed evolution methods described herein). A library of IL-10 recombinants is screened for improved binding to human IL-10 receptor. Library members bound to IL-10R can be recovered by

anti-IL-10R monoclonal antibodies. This screening protocol is likely to result in IL-10 molecules with both antagonistic and agonistic activities. Because initial screen demands for higher affinity, a proportion of the agonists are likely to have improved specific activity when compared to wild-type human IL-10. The

5 functional properties of the mutant IL-10 molecules are determined in biological assays similar to those described above for ultrahigh-affinity IL-10 receptors (cytokine synthesis and MHC class II expression by monocytes, proliferation of B and T cells). An antagonistic IL-4 mutant has been previously generated illustrating the general feasibility of the approach (Kruse et al. (1992) EMBO J.

10 11: 3237-3244). One amino acid mutation in IL-4 resulted in a molecule that efficiently binds to IL-4R  $\alpha$ -chain but has minimal IL-4-like agonistic activity.

Another example of an IL-10 antagonist is IL-20/mda-7, which is a 206 amino acid secreted protein. This protein was originally characterized as mda-7,

15 which is a melanoma cell-derived negative regulator of tumor cell growth (Jiang et al. (1995) Oncogene 11: 2477; (1996) Proc. Nat'l. Acad. Sci. USA 93: 9160). IL-20/mda-7 is structurally related to IL-10, and it antagonizes several functions of IL-10 (Abstract of the 13<sup>th</sup> European Immunology Meeting, Amsterdam, 22-25 June 1997). In contrast to IL-10, IL-20/mda-7 enhances expression of CD80 (B7-

20 1) and CD86 (B7-2) on human monocytes and it upregulates production of TNF- $\alpha$  and IL-6. IL-20/mda-7 also enhances production of IFN- $\gamma$  by PHA-activated PBMC. The invention provides methods of improving genetic vaccines by incorporation of IL-20/mda-7 genes into the genetic vaccine vectors. The methods of the invention can be used to obtain IL-20/mda-7 variants that exhibit improved

25 ability to antagonize IL-10 activity.

When a cytokine antagonist is used as a component of DNA vaccine or gene therapy vectors, maximal local effect is desirable. Therefore, in addition to a soluble form of a cytokine antagonist, a transmembrane form of the antagonist

can be generated. The soluble form can be given in purified polypeptide form to patients by, for example, intravenous injection. Alternatively, a polynucleotide encoding the cytokine antagonist can be used as a component of a genetic vaccine or a gene therapy vector. In this case, either or both of the soluble and transmembrane forms can be used. Where both soluble and transmembrane forms of the antagonist are encoded by the same vector, the target cells express both forms, resulting in maximal inhibition of cytokine function on the target cell surface and in their immediate vicinity.

10           The peptides or polypeptides obtained using these methods can substitute for the natural ligands of the receptors, such as cytokines or other costimulatory molecules in their ability to exert an effect on the immune system via the receptor. A potential disadvantage of administering cytokines or other costimulatory molecules themselves is that an autoimmune reaction could be induced against the natural molecule, either due to breaking tolerance (if using a natural cytokine or other molecule) or by inducing cross-reactive immunity (humoral or cellular) when using related but distinct molecules. Through using the methods of the invention, one can obtain agonists or antagonists that avoid these potential drawbacks. For example, one can use relatively small peptides as agonists that can 20 mimic the activity of the natural immunomodulator, or antagonize the activity, without inducing cross-reactive immunity to the natural molecule. In a presently preferred embodiment, the optimized agonist or antagonist obtained using the methods of the invention is about 50 amino acids or length or less, more preferably about 30 amino acids or less, and most preferably is about 20 amino acids in length, or less. The agonist or antagonist peptide is preferably at least 25 about 4 amino acids in length, and more preferably at least about 8 amino acids in length. Polynucleotides that flank the coding sequence of the mimetic peptide can also be optimized using the methods of the invention in order to optimize the expression, conformation, or activity of the mimetic peptide.

The optimized agonist or antagonist peptides or polypeptides are obtained by generating a library of experimentally generated polynucleotides and screening the library to identify those that encode a peptide or polypeptide that exhibits an enhanced ability to modulate an immune response. The library can be produced using methods such as stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other methods described herein or otherwise known to those of skill in the art. Screening is conveniently conducted by expressing the peptides encoded by the library members on the surface of a population of replicable genetic packages and identifying those members that bind to a target of interest, e.g., a receptor.

The optimized experimentally generated polynucleotides that are obtained using the methods of the invention can be used in several ways. For example, the polynucleotide can be placed in a genetic vaccine vector, under the control of appropriate expression control sequences, so that the mimetic peptide is expressed upon introduction of the vector into a mammal. If desired, the polynucleotide can be placed in the vector embedded in the coding sequence of the surface protein (e.g., geneIII or geneVIII) in order to preserve the conformation of the mimetic. Alternatively, the mimetic-encoding polynucleotide can be inserted directly into the antigen-encoding sequence of the genetic vaccine to form a coding sequence for a "mimotope-on-antigen" structure. The polynucleotide that encodes the mimotope-on-antigen structure can be used within a genetic vaccine, or can be used to express a protein that is itself administered as a vaccine. As one example of this type of application, a coding sequence of a mimetic peptide is introduced into a polynucleotide that encodes the "M-loop" of the hepatitis B surface antigen (HBsAg) protein. The M-loop is a six amino acid peptide sequence bounded by cysteine residues, which is found at amino acids 139-147 (numbering within the S protein sequence). The M-loop in the natural HBsAg protein is recognized by the

monoclonal antibody RFHB7 (Chen et al., Proc. Nat'l. Acad. Sci. USA, 93: 1997-2001 (1996)). According to Chen et al., the M-loop forms an epitope of the HBsAg that is non-overlapping and separate from at least four other HBsAg epitopes.

5

Because of the probable Cys-Cys disulfide bond in this hydrophilic part of the protein, amino acids 139-147 are likely in a cyclic conformation. This structure is therefore similar to that found in the regions of the filamentous phage proteins pIII and pVIII where mimotope sequences are placed. Therefore, one can  
10 insert a mimotope obtained using the methods of the invention into this region of the HBsAg amino acid sequence.

The chemokine receptor CCR6 is an example of a suitable target for a peptide mimetic obtained using the methods. The CCR6 receptor is a 7-  
15 transmembrane domain protein (Dieu et al., Biochem. Biophys. Res. Comm. 236: 212-217 (1997) and J. Biol. Chem. 272: 14893-14898 (1997)) that is involved in the chemoattraction of immature dendritic cells, which are found in the blood and migrate to sites of antigen uptake (Dieu et al., J Exp. Med. 188: 373-386 (1998)). CCR6 binds the chemokine MIP-3 , so a mimetic peptide that is capable of  
20 activating CCR6 can provide a further chemoattractant function to a given antigen and thus promote uptake by dendritic cells after immunization with the antigen antigen-mimetic fusion or a DNA vector that expresses the antigen.

Another application of this method of the invention is to obtain molecules  
25 that can act as an agonist for the macrophage scavenger receptor (MSR; see, Wloch et al., Hum. Gene Ther. 9: 1439-1447 (1998)). The MSR is involved in mediating the effects of various immunomodulators. Among these are bacterial DNA, including the plasmids used in DNA vaccination, and oligonucleotides, which are often potent immunostimulators.



Oligonucleotides of certain chemical structure (e.g., phosphothio-oligonucleotides) are particularly potent, while bacterial or plasmid DNA must be used in relatively large quantities to produce an effect. Also mediated by the MSR  
5 is the ability of oligonucleotides that contain dG residues to stimulate B cells and enhance the activity of immunostimulatory CpG motifs, and of lipopolysaccharides to activate macrophages. Some of these activities are toxic. Each of these immunomodulators, along with a variety of polyanionic ligands, binds to the MSR. The methods of the invention can be used to obtain mimetics of  
10 one or more of these immunomodulators that bind to the MSR with high affinity but are devoid of toxic properties. Such mimetic peptides are useful as immunostimulators or adjuvants.

The MSR is a trimeric integral membrane glycoprotein. The three  
15 extracellular C-terminal cysteine-rich regions are connected to the transmembrane domain by a fibrous region that is composed of an (x-helical coil and a collagen-like triple helix (see, Kodama et al., Nature 343: 531-535 (1990)). Therefore, screening of the library of experimentally generated polynucleotides can be accomplished by expressing the extracellular receptor structure and artificially  
20 attaching it to plastic surfaces. The libraries can be expressed, e.g., by phage display, and screened to identify those that bind to the receptors with high affinity.

The optimized experimentally generated polynucleotides identified by this method can be incorporated into antigen-encoding sequences to evaluate their  
25 modulatory effect on the immune response.

#### 2.6.5.4. COSTIMULATORY MOLECULES CAPABLE OF INHIBITING OR ENHANCING ACTIVATION, DIFFERENTIATION, OR ANERGY OF ANTIGEN-SPECIFIC T CELLS

5 Also provided are methods of obtaining optimized experimentally generated polynucleotides that, when expressed, are capable of inhibiting or enhancing the activation, differentiation, or anergy of antigen-specific T cells. T cell activation is initiated when T cells recognize their specific antigenic peptides in the context of MHC molecules on the plasma membrane of antigen presenting cells (APC),  
10 such as monocytes, dendritic cells (DC), Langerhans cells or B cells. Activation of CD4<sup>+</sup> T cells requires recognition by the T cell receptor (TCR) of an antigenic peptide in the context of MHC class II molecules, whereas CD8<sup>+</sup> T cells recognize peptides in the context of MHC class I molecules.

15 Importantly, however, recognition of the antigenic peptides is not sufficient for induction of T cell proliferation and cytokine synthesis. An additional costimulatory signal, "the second signal", is required. The costimulatory signal is mediated via CD28, which binds to its ligands B7-1 (CD80) or B7-2 (CD86), typically expressed on the antigen presenting cells. In  
20 the absence of the costimulatory signal, no T cell activation occurs, or T cells are rendered anergic. In addition to CD28, CTLA-4 (CD152) also functions as a ligand for B7-1 and B7-2. However, in contrast to CD28, CTLA-4 mediates a negative regulatory signal to T cells and/or to induce anergy and tolerance (Walunas et al. (1994) Immunity 1: 405; Karandikar et al. (1996) J Exp. Med.  
25 184: 783).

B7-1 and B7-2 have been shown to be able to regulate several immunological responses, and they have been implicated to be of importance in the immune regulation in vaccinations, allergy, autoimmunity and cancer. Gene

therapy and genetic vaccine vectors expressing B7-1 and/or B7-2 have also been shown to have therapeutic potential in the treatment of the above mentioned diseases and in improving the efficacy of genetic vaccines.

5           Figure 39 illustrates interaction of APC and CD4<sup>+</sup> T cells, but the same principle is true with CD8<sup>+</sup> T cells, with the exception that the T cells recognize the antigenic peptides in the context of MHC class I molecules. Both B7-1 and B7-2 bind to CD28 and CTLA-4, even though the sequence similarities between these four molecules are very limited (20-30%). It is desirable to obtain mutations  
10 in B7-1 and B7-2 that only influence binding to one ligand but not to the other, or improve activity through one ligand while decreasing the activity through the other. Moreover, because the affinities of B7 molecules to their ligands appear to be relatively low, it would also be desirable to find mutations that improve/alter the activities of the molecules. However, rational design does not enable  
15 predictions of useful mutations because of the complexity of the molecules.

The invention provides methods of overcoming these difficulties, enabling one to generate and identify functionally different B7 molecules with altered relative capacities to induce T cell activation, differentiation, cytokine production,  
20 anergy and/or tolerance. Through use of the methods of the invention, one can find mutations in B7-1 and B7-2 that only influence binding to one ligand but not to the other, or that improve activity through one ligand while decreasing the activity through the other by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is likely to be  
25 the most powerful method in discovering new B7 variants with altered relative binding capacities to CD28 and CTLA-4. B7 variants which act through CD28 with improved activity (and with decreased activity through CTLA-4) are expected to have improved capacity to induce activation of T cells. In contrast, B7 variants which bind and act through CTLA-4 with improved activity (and with

decreased activity through CD28) are expected to be potent negative regulators of T cell functions and to induce tolerance and anergy.

stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-  
5 stochastic polynucleotide reassembly or other reassembly (&/or one or more  
additional directed evolution methods described herein) method is used to  
generate B7 (e.g., B7-1/CD80 and B7-2/CD86) variants which have altered  
relative capacity to act through CD28 and CTLA-4 when compared to wild-type  
B7 molecules. In a preferred embodiment, the different forms of substrate used in  
10 the reassembly (&/or one or more additional directed evolution methods described  
herein) reaction are B7 cDNAs from various species. Such cDNAs can be  
obtained by methods known to those of skill in the art, including RT-PCR-  
Typically, genes encoding these variant B7 molecules are incorporated into  
genetic vaccine vectors encoding an antigen, so that one the vectors can be used  
15 to modify antigen-specific T cell responses. Vectors that harbor B7 genes that  
efficiently act through CD28 are useful in inducing, for example, protective  
immune responses, whereas vectors that harbor genes encoding B7 genes that  
efficiently act through CTLA-4 are useful in inducing, for example, tolerance and  
anergy of allergen- or autoantigen- specific T cells. In some situations, such as in  
20 tumor cells or cells inducing autoimmune reactions, the antigen may already be  
present on the surface of the target cell, and the variant B7 molecules may be  
transfected in the absence of additional exogenous antigen gene. A screening  
protocol that one can use to identify B7-1 (CD80) and/or B7-2 (CD86) variants  
that have increased capacity to induce T cell activation or anergy is diagrammed  
25 herein, and the application of this strategy is described in more detail herein.

Several approaches for screening of the variants can be taken. For  
example, one can use a flow cytometry-based selection systems. The library of  
B7- 1 and B7-2 molecules is transfected into cells that normally do not express

these molecules (e.g., COS-7 cells or any cell line from a different species with limited or no cross- reactivity with man regarding B7 ligand binding). An internal marker gene can be incorporated in order to analyze the copy number per cell. Soluble CTLA-4 and CD28 molecules can be generated to for use in the flow  
5 cytometry experiments. Typically, these will be fused with the Fc portion of IgG molecule to improve the stability of the molecules and to enable easy staining by labeled anti-IgG mAbs, as described by van der Merwe et al. (J. Exp. Med: 185: 393, 1997). The cells transfected with the library of B7 molecules are then stained with the soluble CTLA-4 and CD28 molecules. Cells demonstrating increased or  
10 decreased CTLA- 4/CD28 binding ratio will be sorted. The plasmids are then recovered and the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) B7 variant-encoding sequences identified. These selected B7 variants can then be subjected to new rounds of reassembly (optionally in combination with other directed evolution methods  
15 described herein) and selection, and/or they can be further analyzed using functional assays as described below.

The B7 variants can also be directly selected based on their functional properties. For *in vivo* studies, the B7 molecules can also be evolved to function  
20 on mouse cells. Bacterial colonies with plasmids with mutant B7 molecules are picked and the plasmids are isolated. These plasmids are then transfected into antigen presenting cells, such as dendritic cells, and the capacities of these mutants to activate T cells is analyzed. One of the advantages of this approach is that no assumptions on the binding affinities or specificities to the known ligands  
25 are made, and possibly new activities through yet to be identified ligands can be found. In addition to dendritic cells, other cells that are relatively easy to transfect (e.g., U937 or COS-7) can be used in the screening, provided that the "first T cell signal" is induced by, for example, anti-CD3 monoclonal antibodies. T cell activation can be analyzed by methods known to those of skill in the art,

including, for example, measuring proliferation, cytokine production, CTL activity or expression of activation antigens such as IL-2 receptor, CD69 or HLA-DR molecules. Usage of antigen-specific T, cell clones, such as T cells specific for house dust mite antigen Der p I, will allow analysis of antigen-specific T cell activation (Yssel et al. (1992) J Immunol. 148: 738-745). Mutants are identified that can enhance or inhibit T cell proliferation or enhance or inhibit CTL responses. Similarly variants that have altered capacity to induce cytokine production or expression of activation antigens as measured by, for example, cytokine-specific ELISAs or flow cytometry can be identified.

10

The B7 variants are useful in modulating immune responses in autoimmune diseases, allergy, cancer, infectious disease and vaccination. B7 variants which act through CD28 with improved activity (and with decreased activity through CTLA-4) will have improved capacity to induce activation of T cells. In contrast, B7 variants which bind and act through CTLA-4 with improved activity (and with decreased activity through CD28) will be potent negative regulators of T cell functions and to induce tolerance and anergy. Thus, by incorporating genes encoding these variant B7 molecules into genetic vaccine vectors encoding an antigen, it is possible to modify antigen-specific T cell responses. Vectors that harbor B7 genes that efficiently act through CD28 are useful in inducing, for example, protective immune responses, whereas vectors that harbor genes encoding B7 genes that efficiently act through CTLA-4 are useful in inducing, for example, tolerance and anergy of allergen- or autoantigen-specific T cells. In some situations, such as in tumor cells or cells inducing autoimmune reactions, the antigen may already be present on the surface of the target cell, and the variant B7 molecules may be transfected in the absence of additional exogenous antigen gene.

25

The methods of the invention are also useful for obtaining B7 variants that have increased effectiveness in directing either T<sub>H</sub>1 or T<sub>H</sub>2 cell differentiation. Differential roles have been observed for B7-1 and B7-2 molecules in the regulation of T helper (T<sub>H</sub>) cell differentiation (Freeman et al. (1995) Immunity 2: 523; Kuchroo et al. (1995) Cell 80: 707). T<sub>H</sub> cell differentiation can be measured by analyzing, the cytokine production profiles induced by each particular variant. High levels of IL-4, IL-5 and/or IL-13 are an indication of efficient T<sub>H</sub>2 cell differentiation whereas high levels of IFN- or IL-2 production can be used as a marker of T<sub>H</sub>1 cell differentiation. B7 variants with altered capacity to induce T<sub>H</sub>1 or T<sub>H</sub>2 cell differentiation are useful, for example, in the treatment of allergic, malignant, autoimmune and infectious diseases and in vaccination.

Also provided by the invention are methods of obtaining B7 variants that have enhanced capacity to induce IL-10 production by antigen-specific T cells. Elevated production of IL-10 is a characteristic of regulatory T cells, which can suppress proliferation of antigen-specific CD4<sup>+</sup> T cells (Groux et al. (1997) Nature 389: 737). stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is performed as described above, after which recombinant nucleic acids encoding B7 variants having enhanced capability of inducing IL-10 can be identified by, for example, ELISA or flow cytometry using intracytoplasmic cytokine staining. The variants that induce high levels of IL-10 production are useful in the treatment of allergic and autoimmune diseases.

## 2.6.6. EVOLUTION OF GENETIC VACCINE VECTORS FOR INCREASED VACCINATION EFFICACY AND EASE OF VACCINATION

5           This section discusses the application of the invention to some specific goals in genetic vaccination. Many of these goals relate to improvements in vectors used in vaccine delivery. Unless otherwise indicated the methods are applicable to both viral and nonviral vectors.

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### 2.6.6.1. TOPICAL APPLICATION OF GENETIC VACCINE VECTORS

#### Low efficiency of topical application; protective immune responses have not been demonstrated

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          The invention provides methods of improving the ability of genetic vaccine vectors to induce a desired response after topical application of the vector. Adenoviral vectors topically applied to bare skin have been shown to be capable of acting as vaccine antigen delivery vehicles (Tang et al. (1997) Nature 388: 729-  
20 730). An adenoviral vector that encoded carcinoembryonic antigen (CA) was shown to induce antibodies specific for CA after application to the skin. However, the efficiency of topical application is generally quite low, and protective immune responses have not been demonstrated after topical application.



**Optimizing the topical application efficiency using the methods of the invention**

- 5           The invention provides methods of obtaining vectors that exhibit improved efficiency when topically administered. Several factors can influence topical application efficiency, each of which can be optimized using the methods of the invention. For example, the invention provides methods of improving vector affinity for skin cells, improved skin cell transfection efficiency, improved  
10 persistence of the vector in skin cells (both through improved replication or through avoidance of destruction by immune cells), and improved antigen expression in skin cells, and improved induction of an immune response.

15   **Methods of reassembly (optionally in combination with other directed evolution methods described herein), selection, and screening**

- These methods involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly  
20 using as substrates plasmid, naked DNA vectors, or viral vector nucleic acids, including, for example, adenoviral vectors. Libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids are screened to identify those nucleic acids that confer upon a vector an enhanced ability to induce an immune response upon topical  
25 administration. Screening can be conducted by, for example, topically applying a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) vectors to skin, either mouse skin, monkey skin, or human skin that has been transplanted to immunodeficient mice, or to normal human skin *in vivo*. Vectors that persist and/or provide efficient and

long-lasting expression of marker gene are recovered from the skin samples. In a preferred embodiment, the desired cells are first selected by cell sorting, magnetic beads, or panning. For example, recovery can be effected through expression of a marker gene (e.g., GFP) and detecting cells that are transfected using fluorescence  
5 microscopy or flow cytometry. Cells that express the marker gene can be isolated using flow cytometry based cell sorting. Screening can also involve selection of vectors that induce the highest specific antibody or CTL responses upon administration to a test mammal, or the identification of vectors that provide an enhanced protective immune response to challenge with a corresponding  
10 pathogen. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides are then recovered, e.g., by polymerase chain reaction, or the entire vectors can be purified from these selected cells. If desired, further optimization of topical application efficiency can be obtained by subjecting the recovered experimentally evolved (e.g. by  
15 polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

20 **Administration of genetic vaccine vectors optimized for topical application**

Genetic vaccine vectors that are optimized for topical application can be applied topically to the skin, or by intramuscular, intravenous, intradermal, oral, anal, or vaginal delivery. The vector can be delivered in any of the suitable forms  
25 that are known to those of skill in the art, such as a patch, a cream, as naked DNA, or as a mixture of DNA and one or more transfection-enhancing agents such as liposomes and/or lipids. In preferred embodiments, the genetic vaccine vector is applied after the skin or other target is rendered more susceptible to uptake of the vector by, for example, mechanical abrasion, removal of hair (e.g., by treatment

with a commercially available product such as Nair™, Neet™, and the like). In one embodiment, the skin is pretreated with proteases or lipases to make it more susceptible to DNA delivery. In addition, the DNA can be mixed with the proteases or lipases to enhance gene transfer. Alternatively, a droplet containing the vector and other vaccine components, if any, can simply be administered to the skin.

#### 2.6.6.2. ENHANCED ABILITY TO ESCAPE HOST IMMUNE SYSTEM

##### Limitations of host immune responses directed against the viral vector sometimes even before target cells are entered

Immunogenicity is a particular concern with viral vectors, since a host immune response can prevent a virus from reaching its intended target particularly in repeated administrations. The efficacy of some viral vectors which are used for genetic vaccination and gene delivery is limited by host immune responses directed against the viral vector. For example, most individuals have pre-existing antibodies against adenovirus. Adenoviral vectors can sometimes induce strong immune responses which can destroy cells harboring adenoviral vectors or clear adenoviral vectors from the host even before target cells are entered. Cellular immune responses can also be induced against nonviral vectors administered in naked form or shielded with a coat such as liposomes.

**Methods to create genetic vaccine vectors with improved ability to avoid the humoral and cellular immune systems**

5           The invention provides methods to create genetic vaccine vectors that can escape immune responses that would otherwise be detrimental to obtaining the desired effect. These methods are useful for prolonging expression and secretion of pathogen antigen or pharmaceutically useful protein by genetic vaccine  
10 vaccine vector's ability to avoid the humoral (Ab) and cellular (CTL) immune systems. These strategies can be used in combination to obtain optimal avoidance such as may be required for highly immunogenic vectors such as adenovirus.

15           **Incorporating into genetic vaccines one or more components that inhibit peptide transport and/or MHC class I expression in order to obtain viral vectors that are capable of escaping a host CTL immune response**

          In one embodiment, the invention provides methods of obtaining viral  
20 vectors that are capable of escaping a host CTL immune response. This method can be used in conjunction with methods for obtaining genetic vaccine vectors that can escape the humoral response; the combination of approaches is often desirable, as different viral serotypes often have CTL epitopes in common, suggesting that virus variants which are not recognized by antibodies still are  
25 likely to be recognized by CTLs. This embodiment of the invention involves incorporating into genetic vaccines one or more components that inhibit peptide transport and/or MHC class I expression. An essential element in the activation of cytotoxic T lymphocyte (CTL) responses is an interaction between T cell receptors on CTLs and antigenic peptide-MHC class I molecule complexes on

antigen presenting cells. Expression of MHC class I molecules on thymocytes and antigen presenting cells is a requirement for maturation and activation of antigen-specific CD8<sup>+</sup> T lymphocytes. Thus, genes that encode inhibitors of MHC class I-mediated antigen presentation can be experimentally evolved (e.g. by

5 polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) as described herein and placed into viral vectors to obtain vectors that, when present in target cells, do not induce destruction of the target cells by the cells of the immune system. This can result in prolonged survival of cells harboring genetic vaccine vectors, including those that express a pathogen antigen, as well as

10 vectors that express a pharmaceutically useful protein. In the case of genetic vaccines, reduced expression of MHC class I molecules will allow secretion of the pathogen antigen, which then will be presented by professional antigen presenting cells elsewhere. In the case of vectors encoding pharmaceutical proteins, reduced expression of MHC class I molecules prevents recognition by

15 the immune system prolonging the survival of the cells expressing the gene.

**Reassembly (optionally in combination with other directed evolution methods described herein) genes that encode inhibitors of TAP activity to**

20 **obtain genes that encode optimized TAP inhibitors**

Among the proteins involved in MHC class I molecule expression and antigen presentation are those encoded by TAP genes (transporters associated with antigen processing), which are described above. In one embodiment of the

25 invention, genes that encode inhibitors of TAP activity are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) to obtain genes that encode optimized TAP inhibitors. The substrates for these methods can include, for example, one or more of the viral genes that are known to regulate levels of MHC class I molecule expression. TAP

I and TAP2 gene expression is 5-10-fold and 100-fold reduced, respectively, in cells transformed by adenovirus 12, which results in reduced class I expression and thus leads to reduced virus-specific cytotoxic T lymphocyte responses. Similarly, TAP gene expression is downregulated in 49% of HPV-16<sup>+</sup> cervical carcinomas (Seliger et al. (1997) Immunol. Today 18: 292). Thus, adenovirus and HPV viral nucleic acids provide examples of suitable substrates for carrying out the methods of the invention. Additional examples of suitable stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly substrates for this embodiment of the invention include the human cytomegalovirus (CMV) encoded genes US2, US3 and US 11, which can downregulate MHC class I expression (Wiertz et al. (1996) Nature 384: 432 and Cell (1996) 84: 769; Ahn et al. (1996) Proc. Nat'l. Acad Sci. USA 93: 10990). Another human CMV gene that encodes an inhibitor of TAP-dependent peptide translocation is US6 (Lehner et al. (1997) Proc. Nat'l. Acad Sci. USA 94: 6904-9). Cells transfected with US6 had reduced expression of MHC class I molecules on their surface and reduced capacity to activate cytotoxic T lymphocytes.

**Reassembly (optionally in combination with other directed evolution methods described herein) this 7kb cluster of genes in order to find the most potent sequence for inhibiting the expression of MHC class I molecules, which can also be used for generation of animal models**

Thus, in one embodiment, the invention involves stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of this cluster of genes (approximately 7kb), or fragments thereof, in order to identify the sequences that are most potent in inhibiting the expression of MHC class I molecules. Such optimized TAP

inhibitor polynucleotide sequences are useful not only for use in constructing vectors that can escape CTL immune responses, but also for generation of animal models for use with human viruses that normally are eliminated in laboratory animals due to their immunogenicity. The desired expression levels and functional properties of TAP inhibitors may vary depending on whether genetic vaccine vector, gene therapy vector or animal model is evolved.

**Reassembly (optionally in combination with other directed evolution methods described herein) other genes involved in downregulating expression of MHC class I molecules and/or antigen presentation**

Alternative embodiments of the invention involve stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of other genes that are involved in downregulating expression of MHC class I molecules and/or antigen presentation. Examples of other possible target genes include genes encoding adenoviral E3 protein, herpes simplex ICP47 protein, and tapasin antagonists (Seliger et al. (1997) Immunol. Today 18:292-299; Galoncha et al. (1997) J Exp. Med. 185: 1565-1572; Li et al. (1997) Proc. Nat'l. Acad. Sci. USA 94: 8708-8713; Ortmann et al. (1997) Science 277: 1306-1309.

**A gene that encodes an MHC-like molecule that inhibits NK cell function but is unable to present antigens to T lymphocytes**

Because reduced expression of MHC class I molecules on cell surfaces may act as a stimulus for NK cells, it may be useful to include in genetic vaccine vectors a gene that encodes an MHC like molecule that inhibits NK cell function

but is unable to present antigens to T lymphocytes. An example of such molecule is MHC class I homologue encoded by cytomegalovirus (Farrell et al. (1997) Nature 3 86: 510-514).

5

**Obtaining viral vectors that exhibit an enhanced capability of avoiding attack by CD4<sup>+</sup> T lymphocytes**

The invention also provides methods of obtaining viral vectors that exhibit  
10 an enhanced capability of avoiding attack by CD4<sup>+</sup> T lymphocytes. Such vectors are particularly useful in situations where the target cells are capable of expressing MHC class II molecules, such as in the case of vaccinations and gene therapy targeted to the cells of the immune system. Substrates for stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic  
15 polynucleotide reassembly include genes that encode inhibitors of MHC class II molecules such as, for example, IL-10 and antagonists of IFN- (such as soluble IFN- receptor).

20 **Improving sequences that result in inhibition of MHC class I expression, MHC class II expression, and additional sequences that encode homologs of MHC class I molecules**

Vectors that have the greatest capability of escaping the host immune  
25 system, will typically include DNA sequences that result in inhibition of MHC class I expression and MHC class II expression, and additional sequences that encode homologs of MHC class I molecules. The properties of all these can be further improved by stochastic (e.g. polynucleotide shuffling & interrupted



synthesis) and non-stochastic polynucleotide reassembly according to the methods of the invention.

5    **Methods for screening the library to identify those polynucleotides that exhibit the desired effect on the host immune response**

Once a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) DNA molecules is  
10    obtained, any of several methods are available for screening the library to identify those polynucleotides that, when present in a viral vector (or in an animal model) exhibit the desired effect on the host immune response. For example, to obtain experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that inhibit MHC class I expression  
15    and/or antigen presentation, a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes can be incorporated into genetic vaccine or gene therapy vectors and transfected into human cell lines, such as, for example, HeLa, U937 or Jijoye, in a single tube transfection. Primary human monocytes, or dendritic cells generated by culturing  
20    human cord blood cells or monocytes in the presence of IL-4 and GM-CSF, are also suitable. Initial screening can be done using FACS-sorting.

25    **Cells expressing the lowest levels of MHC class I molecules are expected to have the lowest capacity to induce CTL responses**

Cells expressing the lowest levels of MHC class I molecules are selected, the polynucleotides that encode the MHC inhibitors, or whole plasmids containing the sequences, are recovered. If desired, the selected sequences can be

subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection. Cells expressing the lowest levels of MHC class I molecules are expected to have the lowest capacity to induce CTL responses.

5

**Screening method: injecting library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that encode inhibitors of MHC class I expression incorporated into HPV vectors**

10

Another screening method involves incorporating libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that encode inhibitors of MHC class I expression are incorporated into human papillomavirus (HPV) vectors. This library is injected into the skin of mice.

15

**Normally, murine cells expressing HPV are destroyed by the host immune system. Cells expressing potent inhibitors of peptide transportation and/or MHC class expression will be able to escape the immune response**

20

However, cells expressing potent inhibitors of peptide transportation and/or MHC class expression will be able to escape the immune response. The cells that express a marker gene present on the vector, such as GFP, for extended periods of time are selected, the sequences or whole plasmids are recovered, and, if further optimization is desired, the selected sequences are subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection. Long- lasting maintenance of HPV in

25

mice will allow drug screening and vaccine studies, which to date have not been possible due to high immunogenicity of HPV in mice.

- 5    **Evolved inhibitors will block efficient presentation of immunogenic peptides, and hence, will strongly downregulate activation of antigen-specific CTLs allowing long-lasting transgene expression *in vivo***

- 10            In another embodiment, the libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides encoding inhibitors of MHC class I expression are incorporated into human adenovirus vectors. This library is transfected into human cell lines, such as HeLa cells, and cells expressing the lowest levels of MHC class I molecules are selected as described above. The sequences that provide the lowest
- 15    levels of MHC class I expression are further tested by analyzing the capacity of antigen-presenting cells transfected with adenovirus harboring evolved inhibitors of MHC class I expression to activate specific T cell lines or clones. These inhibitors will block efficient presentation of immunogenic peptides, and hence, will strongly downregulate activation of antigen-specific CTLs allowing long-
- 20    lasting transgene expression *in vivo*.

#### **Methods to screen for inhibitors**

- 25            Methods to screen for improved inhibitors of MHC class II expression include detection of MHC class II molecules on the surface of the target cells by fluorescent labeled specific monoclonal antibodies, fluorescence microscopy, and flow cytometry. In addition, the inhibitors can be analyzed in functional assays by studying the capacity of the inhibitors to block activation of MHC class II

restricted antigen- specific CD4<sup>+</sup> T lymphocytes. For example, one can determine the capacity of the inhibitor to inhibit induction of CD4<sup>+</sup> T cell proliferation induced by autologous antigen presenting cells, such as monocytes, dendritic cells, B cells or EBV-transformed B cell lines, that harbor genes encoding the MHC class II inhibitor or have been treated with supernatant containing the inhibitor.

### 2.6.6.3. ENHANCED ANTIVIRAL ACTIVITY

#### Obtaining a recombinant viral vector which has an enhanced ability to induce an antiviral response in a cell

The invention also provides methods of obtaining a recombinant viral vector which has an enhanced ability to induce an antiviral response in a cell. These methods can include the steps of:

- (1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprise a viral vector, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant viral vectors;
- (2) transfecting the library of recombinant viral vectors into a population of mammalian cells;
- (3) staining the cells for the presence of Mx protein; and
- (4) isolating recombinant viral vectors from cells which stain positive for Mx protein, wherein recombinant viral vectors from positive staining cells exhibit enhanced ability to induce an antiviral response.

Stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is used to produce a library of recombinant

viral vectors. The library is transfected into a population of mammalian cells, which are then tested for ability to induce an antiviral response. One suitable test involves staining the cells for the presence of Mx protein, which is produced by cells that are exhibiting an antiviral response (see, e.g., Hallinen et al. (1997) Pediatric Research 41: 647-650; Melen et al. (1994) J Biol. Chem. 269: 2009-2015).

Recombinant viral vectors can be isolated from cells which stain positive for Mx protein. These recombinant viral vectors from positive staining cells are enriched for those that exhibit enhanced ability to induce an antiviral response. Viral vectors for which this method is useful include, for example, influenza virus.

#### 2.6.6.4. EVOLUTION OF VECTORS HAVING INCREASED COPY NUMBER IN PRODUCTION CELLS

Desirability of method to increase the plasmid copy number after all elements have been cloned in the vector, especially when the plasmid is to be manufactured on a large scale

The invention provides methods for obtaining vector components that, when present in a genetic vaccine vector (such as a plasmid) the ability to replicate to a high copy number in a cell used to produce the vector. Plasmids can incorporate various heterologous DNA sequences, however the size or the nature of the cloned sequences in a given plasmid vector may render that vector less able to grow to high copy number in the bacteria in which it is propagated. It is therefore desirable to have a method to increase the plasmid copy number after all elements have been cloned into the vector. This is especially important when the

plasmid is to be manufactured on a large scale as will be the case for genetic vaccines.

5    **Incorporating into the plasmid one or more polynucleotide sequences that bind proteins which would otherwise be toxic to the bacterium**

          The methods of the invention involve incorporating into the plasmid one or more polynucleotide sequences that bind proteins which would otherwise be  
10   toxic to the bacterium. One suitable toxic moiety and binding site combination is the transcription factor GATA-1 and its recognition site. It has been shown that expression of a DNA-binding fragment of GATA-1 is toxic to bacteria; this toxicity apparently results from inhibition of bacterial DNA replication. Trudel et al. ((1996) Biotechniques 20: 684- 693) have described a plasmid (pGATA) that  
15   expresses the Z2B2 region of GATA-1 as a GST fusion protein. The expression of the fusion protein in this plasmid is under the control of the IPTG-inducible lac promoter. The GST-GATA-1 fragment also binds strongly to a sequence from the mouse  $\gamma$ -globin gene promoter as well as to the C-oligonucleotide from the  $\gamma$ -globin gene 3' enhancer; either or both of these are suitable for use as binding  
20   sites in the methods of the invention.

- Including only a single form of the selectable marker in the shuffling reaction to achieve significant diversity in the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library to recover a plasmid which is improved in its growth properties while fully retaining the appropriate selection function of the plasmid

The plasmids preferably also include a selectable marker such as, for example, kanamycin resistance (aminoglycoside 3'-phosphotransferase (EC 2.7.1.95)) and the like. The plasmid backbone polynucleotide sequence is subjected to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly as described herein to generate a library of plasmids which have different backbone sequences and possibly different supercoil densities. In order to introduce sufficient sequence diversity to search for improved function, it is preferable to perform family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly. This can be accomplished in the context of the present invention by including in the reassembly (optionally in combination with other directed evolution methods described herein) reaction(s) only a single form of the selectable marker. In this way, significant diversity can be achieved in the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library to recover a plasmid which is improved in its growth properties while fully retaining the appropriate selection function of the plasmid.

### Selecting for high copy number plasmids

The selection for high copy number plasmids is performed by introducing  
5 the library of experimentally evolved (e.g. by polynucleotide reassembly &/or  
polynucleotide site-saturation mutagenesis) recombinant plasmids into the desired  
host cell. The host cells also express the toxic moiety, preferably under the control  
of a promoter which is inducible. For example, the pGATA plasmid is suitable for  
use in E. coli host cells. The experimentally evolved (e.g. by polynucleotide  
10 reassembly &/or polynucleotide site-saturation mutagenesis) plasmids are  
introduced into the cells under non-inducing conditions. Transformed cells are  
then placed under conditions which induce expression of the toxic moiety. For  
example, E. coli cells that contain pGATA can be placed on media containing  
increasing concentrations of IPTG. Those target plasmids which grow to high  
15 copy number in the bacteria will express correspondingly higher numbers of the  
binding sequences for GATA-1. The target plasmids will bind the GST-GATA-1  
fusion protein and thus neutralize the toxic effects on the bacteria.

Plasmids with the highest copy number are detected as those which confer the  
best growth to bacteria on the inducer-containing growth media. Such plasmids  
20 can be recovered and transformed into bacteria which lack the gene that encodes  
the toxic moiety; these plasmids should retain their high copy number  
characteristics. Further rounds of reassembly (optionally in combination with  
other directed evolution methods described herein) can be used to isolate high  
copy number plasmids by the above selection procedure. Alternatively, manual  
25 screening can be done in the bacterial host of choice, lacking the toxic moiety-  
encoding plasmid, to avoid any effects due to the presence of this extraneous  
plasmid.



## 2.7. OPTIMIZATION OF TRANSPORT AND PRESENTATION OF ANTIGENS

5           The invention also provides methods of obtaining genetic vaccines and accessory molecules that can improve the transport and presentation of antigenic peptides. A library of experimentally generated polynucleotides is created and screened to identify those that encode molecules that have improved properties compared to the wild-type counterparts. The polynucleotides themselves can be  
10       used in genetic vaccines, or the gene products of the polynucleotides can be utilized for therapeutic or prophylactic applications.

### 2.7.1. PROTEASOMES

15           The class I peptides presented on major histocompatibility complex molecules are generated by cellular proteasomes. Interferon-gamma can stimulate antigen presentation, and part of the mechanism of action of interferon may be due to induction of the proteasome beta-subunits LMP2 and LMP7, which replace  
20       the homologous beta-subunits Y (delta) and X (epsilon). Such a replacement changes the peptide cleavage specificity of the proteasome and can enhance class I epitope immunogenicity. The Y (delta) and X (epsilon) subunits, as well as other recently discovered proteasome subunits such as the MECL-1 homologue MC14, are characteristic of cells which are not specialized in antigen presentation. Thus,  
25       the incorporation into cells by DNA transfer of LMP2, LMP7, MECL-1 and/or other epitope presentation-specific and potentially interferon-inducible subunits can enhance epitope presentation. It is likely that the peptides generated by the proteasome containing the interferon-inducible subunits are transported to the endoplasmic reticulum by the TAP molecules.

The invention provides methods of obtaining proteasomes that exhibit increased or decreased ability to specifically process MHC class I epitopes. According to the methods, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is used to obtain evolved proteins that can either have new specificities which might enhance the immunogenicity of some proteins and/or enhance the activity of the subunits once they are bound to the proteasome. Because the transition from a non-specific proteasome to a class I epitope-specific proteasome can pass through several states (in which some but not all of the interferon-inducible subunits are associated with the proteasome), many different proteolytic specificities can potentially be achieved. Evolving the specific LMP-like subunits can therefore create new proteasome compositions which have enhanced functionality for the presentation of epitopes.

15

The methods involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly using as substrates two or more forms of polynucleotides which encode proteasome components, where the forms of polynucleotides differ in at least one nucleotide. reassembly (optionally in combination with other directed evolution methods described herein) is performed as described herein, using polynucleotides that encode any one or more of the various proteasome components, including, for example, LMP2, LMP7, MECL-1 and other individual proteasome components that are specifically involved in class I epitope presentation. Examples of suitable substrates are described in, e.g., Stoliwasser et al. (1997) Eur. J Immunol. 27: 1182- 1187 and Gaczynska et al. (1996) J Biol. Chem. 271: 17275-17280. In a preferred embodiment, polynucleotide reassembly (optionally in combination with other directed evolution methods described herein) is used, in which the

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different substrates are proteasome component-encoding polynucleotides from different species.

After the reassembly (&/or one or more additional directed evolution  
5 methods described herein) reaction is completed, the resulting library of  
experimentally generated polynucleotides is screened to identify those which  
encode proteasome components having the desired effect on class I epitope  
production. For example, the experimentally generated polynucleotides can be  
introduced into a genetic vaccine vector which also encodes a particular antigen  
10 of interest. The library of vectors can then be introduced into mammalian cells  
which are then screened to identify cells which exhibit increased antigen- specific  
immunogenicity. Methods of analyzing proteasome activity are described in, for  
example, Groettrup et al. (1997) Proc. Nat'l. Acad. Sci. USA 94: 8970- 8975 and  
Groettrup et al. (1997) Eur. J. Immunol. 26: 863-869.

15

Alternatively, one can use the methods of the invention to evolve proteins  
which bind strongly to the proteasome but have decreased or no activity, thus  
antagonizing the proteasome activity and diminishing a cells ability to present  
class I molecules. Such molecules can be applied to gene therapy protocols in  
20 which it is desirable to lower the immunogenicity of exogenous proteins  
expressed in the cells as a result of the gene therapy, and which would otherwise  
be processed for class I presentation allowing the cell to be recognized by the  
immune system. Such high-affinity low-activity LMP- like subunits will  
demonstrate immuno suppressive effects which are also of use in other therapeutic  
25 protocols where cells expressing a non-self protein need to be protected from an  
immune response.

The specificity of the proteasome and the TAP molecules (discussed below) may  
have co-evolved naturally. Thus it may be important that the two pathways of the

class I processing system be functionally matched. A further aspect of the invention involves performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly simultaneously on the two gene families followed by random combinations of the two in order to discover appropriate matched proteolytic and transport specificities.

### 2.7.2. ANTIGEN TRANSPORT

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The invention provides methods of improving transport of antigenic peptides from the cytosolic compartment to the endoplasmic reticulum, and thereby to the cell surface in the context of MHC class I molecules. Enhanced expression of antigenic peptides results in enhanced immune response, particularly in improved activation of CD8<sup>+</sup> cytotoxic lymphocytes. This is useful in the development of DNA vaccines and in gene therapy.

In one embodiment, the invention involves evolving TAP-genes (transporters associated with antigen processing) to obtain genes that exhibit improved antigen presentation. TAP genes are members of ATP-binding cassette family of membrane translocators. These proteins transport antigenic peptides to MHC class I molecules and are involved in the expression and stability of MHC class I molecules on the cell surface. Two TAP genes, TAP1 and TAP2, have been cloned to date (Powis et al. (1996) Proc. Nat'l. Acad. Sci. USA 89: 1463-1467; Koopman et al. (1997) Curr. Opin. Immunol. 9: 80-88; Monaco (1995) J Leukocyte Biol. 57: 543-57). TAP1 and TAP2 form a heterodimer and these genes are required for transport of peptides into the endoplasmic reticulum, where they bind to MHC class I molecules. The essential role of TAP gene products in presentation of antigenic peptides was demonstrated in mice with disrupted TAP

genes. TAP1-deficient mice have drastically reduced levels of surface expression of MHC class I, and positive selection of CD8<sup>+</sup> T cells in the thymus is strongly reduced. Therefore, the number of CD8<sup>+</sup> T lymphocytes in the periphery of TAP-deficient mice is extremely low. Transfection of TAP genes back into these cells  
5 restores the level of MHC class I expression.

TAP genes are a good target for polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein) because of natural polymorphism  
10 and because these genes of several mammalian species have been cloned and sequenced, including human (Beck et al. (1992) J Mol. Biol. 228: 433-441; Genbank Accession No. Y13582; Powis et al., supra.), gorilla TAP1 (Laud et al. (1996) Human Immunol. 50: 91-102), mouse (Reiser et al. (1988) Proc. Nat'l. Acad. Sci. USA 85: 2255- 2259; Marusina et al. (1997) J Immunol, 158: 5251-  
15 5256, TAP1: Genbank Accession Nos. U60018, U60019, U60020, U60021, U60022, and L76468-L67470; TAP2: Genbank Accession Nos. U60087, U60088, U6089, U60090, U60091 and U60092), hamster (TAP1, Genbank Accession Nos. AF001154 and AF001157; TAP2, Genbank Accession Nos. AF001 156 and AF001155). Furthermore, it has been shown that point mutations in TAP genes  
20 may result in altered peptide specificity and peptide presentation. Also, functional differences in TAP genes derived from different species have been observed. For example, human TAP and rat TAP containing the rTA.P2a allele are rather promiscuous, whereas mouse TAP is restrictive and select against peptides with C-terminal small polar/hydrophobic or positively charged amino acids. The basis  
25 for this selectivity is unknown.

The methods of the invention involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of TAP1 and TAP2 genes using as substrates at least

two forms of TAP1 and/or TAP2 polynucleotide sequences which differ in at least one nucleotide position. In a preferred embodiment, TAP sequences derived from several mammalian species are used as the substrates for reassembly (optionally in combination with other directed evolution methods described herein).

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Natural polymorphism of the genes can provide additional diversity of substrate. If desired, optimized TAP genes obtained from one round of reassembly (optionally in combination with other directed evolution methods described herein) and screening can be subjected to additional reassembly (optionally in  
10 combination with other directed evolution methods described herein)/screening rounds to obtain further optimized TAP-encoding polynucleotides.

To identify optimized TAP-encoding polynucleotides from a library of recombinant TAP genes, the genes can be expressed on the same plasmid as a  
15 target antigen of interest. If this step is limiting the extent of antigen presentation, then enhanced presentation to CD8<sup>+</sup> CTL will result. Mutants of TAPs may act selectively to increase expression of a particular antigen peptide fragment for which levels of expression are otherwise limiting, or to cause transport of a peptide that would normally never be transferred into the RER and made available  
20 to bind to MHC Class I.

When used in the context of gene therapy vectors in cancer treatment, evolved TAP genes provide a means to enhance expression of MHC class I molecules on tumor cells and obtain efficient presentation of antigenic tumor-  
25 specific peptides. Thus, vectors that contain the evolved TAP genes can induce potent immune responses against the malignant cells. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) TAP genes can be transfected into malignant cell lines that express low levels of MHC class I molecules using retroviral vectors or electroporation.